L Number	Hits	Search Text	DB	Time stamp
1	487	recombinant same (mhc or hla) same (antibod$3 or immunoglobulin)	USPAT; US-PGPUB; EPO; DERWENT	2003/04/01 12:19
2	12	recombinant adj (mhc or hla) same (antibod$3 or immunoglobulin)	USPAT; US-PGPUB; EPO; DERWENT	2003/04/01 12:20

L7 ANSWER 1 OF 21 CAPLUS COPYRIGHT 2003 ACS DUPLICATE 1

TI Method and kit for detecting **antibodies** to major histocompatibility complex antigens

AB The invention provides a method of detecting the presence of anti-MHC **antibodies** in a sample comprising contacting said sample with one or more **recombinant MHC** mols. or functionally equiv. variants, derivs. or fragments thereof and detecting the binding or absence of binding of **antibodies** to said **recombinant MHC** mols. This method allows the detection and/or identification of one or more specific MHC particularly HLA allele **antibodies**. Serum samples were analyzed using HLA-coated Combiplates. Bound **antibodies** were detd. using anti-human IgG antiserum conjugated to horseradish peroxidase.

SO U.S. Pat. Appl. Publ., 14 pp.
CODEN: USXXCO

IN Barnardo, Martin C.; Harmer, Andrea W.; Bunce, Michael; Vaughn, Robert W.; Welsh, Kenneth I.

6127524 presenting peptides

fused MHC

WO 96/04314

ANSWER 12 OF 21 SCISEARCH COPYRIGHT 2003 ISI (R)

TI In vitro induction of specific cytotoxic T lymphocytes using recombinant single-chain MHC class I peptide complexes

AB We have previously described the production and purification of a murine single-chain, soluble recombinant major histocompatibility complex (MHC) class I molecule (SC-Kd). A similar strategy was devised to produce a recombinant HLA-A2.1 (SC-A2) molecule. The latter was composed of the first three domains of the HLA-A2.1 heavy chain connected to human beta(2)-microglobulin through a spacer of 15 amino acids. Immunoaffinity-purified SC-A? molecules were correctly folded and biologically functional. They specifically bound HLA-A2-restricted peptides and induced a peptide-specific cytotoxic T lymphocyte (CTL) clone to proliferate and secrete interleukin-2. The ability of murine and human SC-MHC molecules to elicit primary CTLs in vitro was next investigated. When coated in high density onto beads, complexes of antigenic peptide and SC-Kd or SC-A2 molecules efficiently induced a specific primary CTL response in vitro. Furthermore, the structural features of these CTLs were characterized by T cell receptor-P chain analysis, which revealed rearrangements very similar, if not identical, to those found in CTLs generated by in vivo immunization. Such single-chain, soluble **recombinant MHC** class I molecules should provide a useful tool in particular for peptide binding assays and for in vitro primary CTL induction to identify immunogenic peptides such as those derived from known tumor-associated antigens.

SO JOURNAL OF IMMUNOTHERAPY, (JUL 1998) Vol. 21, No. 4, pp. 283-294.
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ISSN: 1053-8550.

AU Lone Y C; Motta I; Mottez E; Guilloux Y; Lim A; Demay F; Levraud J P; Kourilsky P; Abastado J P (Reprint)

ANSWER 9 OF 21 SCISEARCH COPYRIGHT 2003 ISI (R)

TI A single-chain fusion molecule consisting of peptide, major histocompatibility gene complex class I heavy chain and beta(2)-microglobulin can fold partially correctly, but binds peptide inefficiently

AB The function of major histocompatibility complex class I (MHC-I) molecules is to sample peptides from the intracellular environment and present these peptides to CD8(+) cytotoxic T lymphocytes (CTL). We have attempted to develop a general approach to produce large amounts of pure and active **recombinant MHC**-I molecules. A convenient source of MHC-I molecules would be a valuable tool in structural and biochemical analysis of MHC-I, and in experiments using MHC-I molecules to enable specific manipulations of experimental and physiological CTL responses. Here we describe the generation of a recombinant murine MHC-I molecule, which could be produced in large amounts in bacteria. The **recombinant MHC**-I protein was expressed as a single molecule (PepSc) consisting of the antigenic peptide linked to the MHC-I heavy chain and further linked to human beta(2)-microglobulin (h beta(2)m). The PepSc molecule was denatured, extracted, purified and folded using a recently developed in vitro reiterative refolding strategy. This led to the formation of soluble, **recombinant MHC** -I molecules, which migrated as monomers of the expected size when submitted to non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Serological analysis revealed the presence of some, but not all, MHC-I-specific epitopes. Biochemically, PepSc could bind peptide, however, rather ineffectively. We suggest that a partially correctly refolded MHC-I has been obtained.

SO SCANDINAVIAN JOURNAL OF IMMUNOLOGY, (OCT 1999) Vol. 50, No. 4, pp. 355-362.
Publisher: BLACKWELL SCIENCE LTD, P O BOX 88, OSNEY MEAD, OXFORD OX2 0NE, OXON, ENGLAND.
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AU SylvesterHvid C; Nielsen L L B; Hansen N J V; Pedersen L O; Buus S (Reprint)

DETECTION OF HLA-SPECIFIC IgG USING SINGLE RECOMBINANT HLA ALLELES

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Screening for HLA specific antibodies is a complex procedure and it can be difficult to define all the HLA specificities to which antibody has been produced in some patients, particularly those with antibodies to multiple epitopes. Also antibodies binding *in vitro* to lymphocytes may be directed at non-HLA targets. The more recent use of isolated HLA class I and class II antigens from cell lines with ELISA or flow cytometric techniques has removed the confounding factor of non-HLA molecules. However the soluble antigen preparations contain a minimum of 1 A locus, 1 B locus and 1 Cw locus antigen and may have as many as 2 of each of these. Each antigen must therefore be tested at least twice, and usually many more times, in different combinations with other antigens in order to determine which antigen in any preparation is the target. In this study we have investigated the use of soluble HLA molecules of a single allele for antibody screening.

The use of recombinant biotinylated HLA molecules bound onto streptavidin to form tetrameric complexes has been described. Two preparations of biotinylated HLA-A*0201 monomers, each presenting a different peptide, were bound in saturation to streptavidin microspheres. The coated beads were incubated with serum previously shown a) to be specific for HLA-A2 epitopes, b) to have no HLA-specific antibodies or c) to have class I specific antibodies other than anti-A2. Antibody binding to the beads was measured by use of a FITC conjugated anti-human IgG antibody and flow cytometric analysis. All 38 A2 specific sera, and 1 of 15 non-A2 specific sera exhibited binding in the positive range demonstrating very high specificity and sensitivity. The nature of the presented peptide did not appreciably affect the antibody binding.

One A2 monomer/peptide combination was also coupled to streptavidin-coated 96-well plates and an ELISA was employed to detect specific antibody. Preliminary results using 24 anti-A2 positive and negative sera showed 100% concordance with the cytotoxically defined specificities.

These early results demonstrate the potential for a revolutionary anti-HLA screening technique which would facilitate antibody analysis and allow hitherto unattainable specificity definition. We shall be testing more recombinant class I molecules and eventually aim for a comprehensive coverage of all class I and II alleles.

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Brief communication

A new murine lymphocytotoxic monoclonal antibody recognizing HLA-A2, -A28 and -A9

S. Mizuno, F. Yako, H. Ohta, T. Kato, K. Wada, C. Uchigiri, M. Furuta, K. Ohya, T. Kurachi, T. Murase, T. Kamiya, K. Ozawa. A new murine lymphocytotoxic monoclonal antibody recognizing HLA-A2, -A28 and -A9. Tissue Antigens 1996: 48: 224–227. © Munksgaard, 1996

Monoclonal antibodies recognizing polymorphic as well as monomorphic epitopes on HLA antigens are important tools for understanding the immunobiology of HLA molecules. We immunized BALB/c mice with a HLA-A2 transfectant and screened for hybridomas which reacted with a HLA-A2 transfectant but not with a HLA-B75 transfectant. After subcloning by limiting dilution four times, a hybridoma secreting a monoclonal antibody (mAb) (IgG 2a, kappa) designated 1-145 was established. 1-145 reacted with Epstein-Barr virus transformed B lymphoblastoid cell lines (B cell lines) which expressed HLA-A2, -A28, -A23 and -A24. The titer of 1-145 in culture supernatant against HLA-A2 and -A28 antigens was similar and the titer against HLA-A23 was lower. 1-145 reacted with cells expressing HLA-A24 but the titer against HLA-A24 antigens was even lower than that againt HLA-A23 antigens. The HLA-A24 antigens on the peripheral blood lymphocytes were not detected by 1-145 possibly due to the lower expression compared to the B cell lines. These differences of the titers were reflected to microlymphocytotoxicity assay in which 1-145 culture supernatant lysed all PBLs expressing HLA-A2,-A28 and -A23 but did not lyse PBLs expressing HLA-A24. Published deduced amino acid sequence data of HLA class I molecules indicate that Lys in position 127 may be critical for 1-145 binding.

S. Mizuno, F. Yako, H. Ohta, T. Kato, K. Wada, C. Uchigiri, M. Furuta, K. Ohya, T. Kurachi, T.Murase, T. Kamiya and K. Ozawa

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Key words: HLA-A cross reactive group – HLA typing – murine monoclonal antibody

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The development of mAb to HLA antigens has provided useful tools for understanding the immunobiology of the polymorphic molecules. In this report, we describe a murine cytotoxic mAb designated 1-145 which recognizes HLA-A2, -A28, -A23 and -24.

1-145 was produced by fusing murine myloma cells, P3-X63-Ag8.653 with spleen cells from a BLAB/c mouse immunized with a murine fibroblast cell line (a generous gift form Dr. Soo Young Yang, Sloan Kettering Cancer Center, New York) which expresses human beta2-microglobulin and HLA-A2 (A*0201) on the cell surface. A clone which produced antibody reactive with the HLA-A2 transfectant but not reactive with HLA-B75 transfectant (a cDNA encoding B*1502 was a generous gift from Dr. Peter Parham, Stanford University, Stanford) was selected and subcloned four times to ensure monoclonality. An isotype of the 1-145 mAb was IgG2a, kappa which fixes complement. Flow cytometry analysis of 1-145 showed that fluorescence intensities of HLA-A2, -A28 and -A23 expressing B cell lines were comparable to those of W6/32 stained cells. In contrast, the mean fluorescence intensity of HLA-A24 expressing cells (TISI) stained with 1-145 was 1/16th of that of the cells stained with W6/32 (Fig.1A). Fluorescence intensity of HLA-A23 expressing PBL was lower than those of HLA-A2 and -A28 expressing PBLs. There was no reactivity against HLA-A24 expressing PBL (Fig. 1B).

The reactivity of 1-145 was also examined on various homozygous B cell lines using PA assay. 1-145 reacted strongly with B cell lines expressing HLA-A2, -A28 and -A23, in contrast 1-145 reacted weakly with B cell lines expressing HLA-A24. The titer of 1-145 mAb against the HLA-A24 expressing cells were significantly less than those against the HLA-A2, -A28 and -A23 expressing cells. No reactivity was observed against the cells expressing

other HLA-A antigens (Table 1). The reactivity of 1-145 against a panel of PBLs was examined by PA assay and LCT. 1-145 reacted with PBLs expressing HLA-A2,-A28 and -A23 but the titer against HLA-A23 expressing cells were significantly lower. No reactivity was observed against HLA-A24 express-

Table 1.
Reactivity of mAb 1-145 with a panel of B-lymphoblastoid cell lines and PBLs

B cell line	HLA-A allele or phenotype	PA titer	PBLs ID	HLA-A phenotype	PA titer	LCT titer
JESTHOM	A*0201	16000	R6073	A2	16000	128
RML	A*0204	16000	R6058	A2, A11.1	8000	128
AMA1	A*6802	8000	R6063	A2, A26	2000	256
WT51	A*2301	16000	23399	A28	4000	256
TISI	A*2402	80	23398	A28, A1	4000	256
KAS-116	A*2402	80	23591	A28, A1	4000	256
E4181324	A*0101	0	19110	A23, A33	16	1
HOM-2	A*0301	0	481	A23, A29	16	4
WT100-BIS	A11	0	R6040	A24, A11.1	0	0
BM92	A*2501	0	R6049	A24, A33	0	0
DEU	A*3101	0	R6086	A24, A33	0	0
WT47	A*3201	0				
HOR	A33	0				

B-lymphoblastoid cell lines were obtained through the 10th International Histocompatibility Workshop (1). HLA-A alleles were shown when available (2). A standard lymphocytotoxicity test (LCT) and an indirect immunorosetting assay (PA assay) (3) were used. "0" indicates no reactivity.

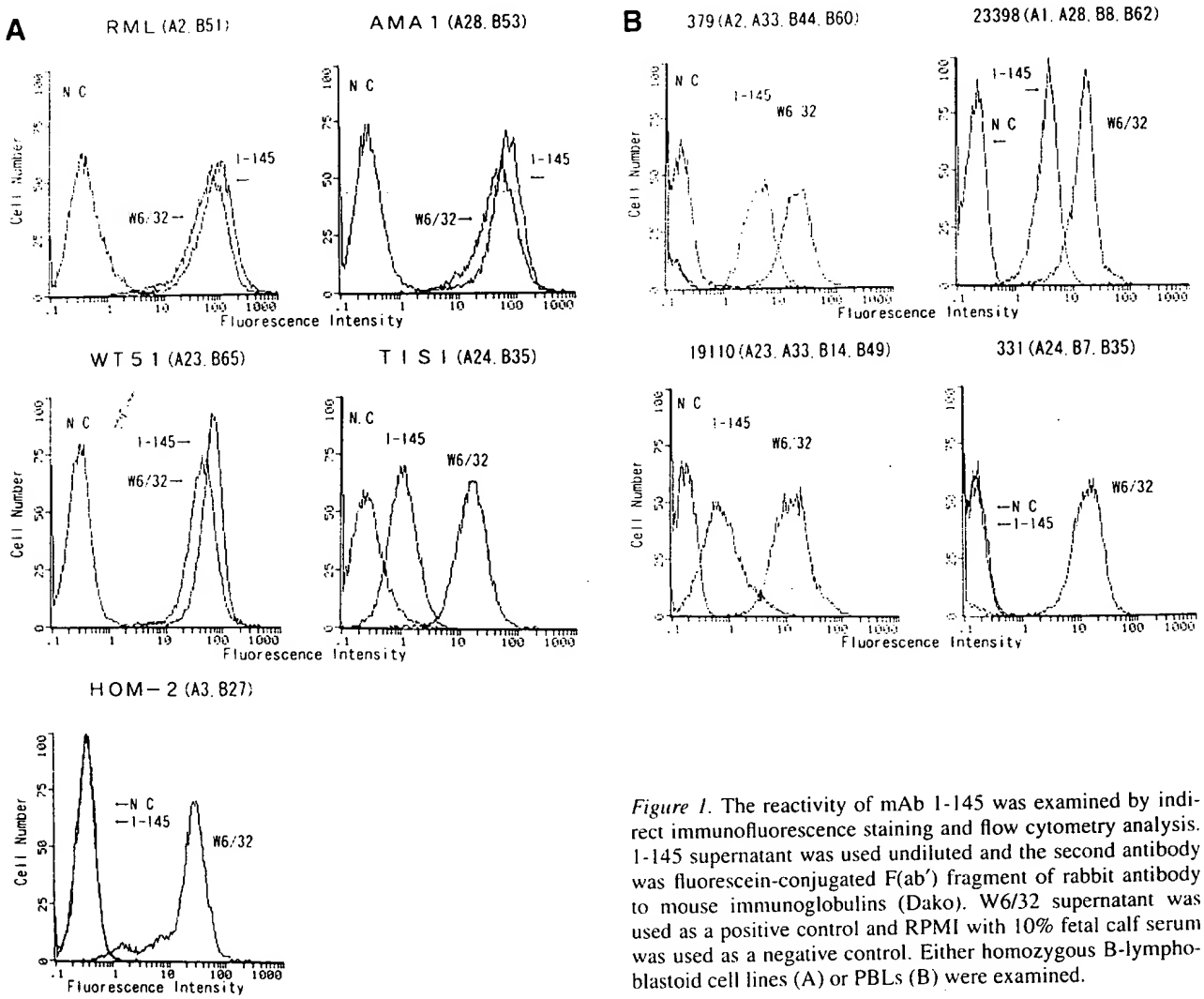

Figure 1. The reactivity of mAb 1-145 was examined by indirect immunofluorescence staining and flow cytometry analysis. 1-145 supernatant was used undiluted and the second antibody was fluorescein-conjugated F(ab') fragment of rabbit antibody to mouse immunoglobulins (Dako). W6/32 supernatant was used as a positive control and RPMI with 10% fetal calf serum was used as a negative control. Either homozygous B-lymphoblastoid cell lines (A) or PBLs (B) were examined.

ing cells (Table 1). The difference of the reactivity between the B cell lines and the PBLs may be due to the higher expression of HLA class I antigens on the homozygous B cell lines compared to the PBLs (Fig. 1) (4). To confirm the reactivity of 1-145 against PBLs, a total of 315 locally collected HLA-typed PBLs were examined by LCT. 1-145 reacted strongly (score 8) with PBLs expressing HLA-A2, -

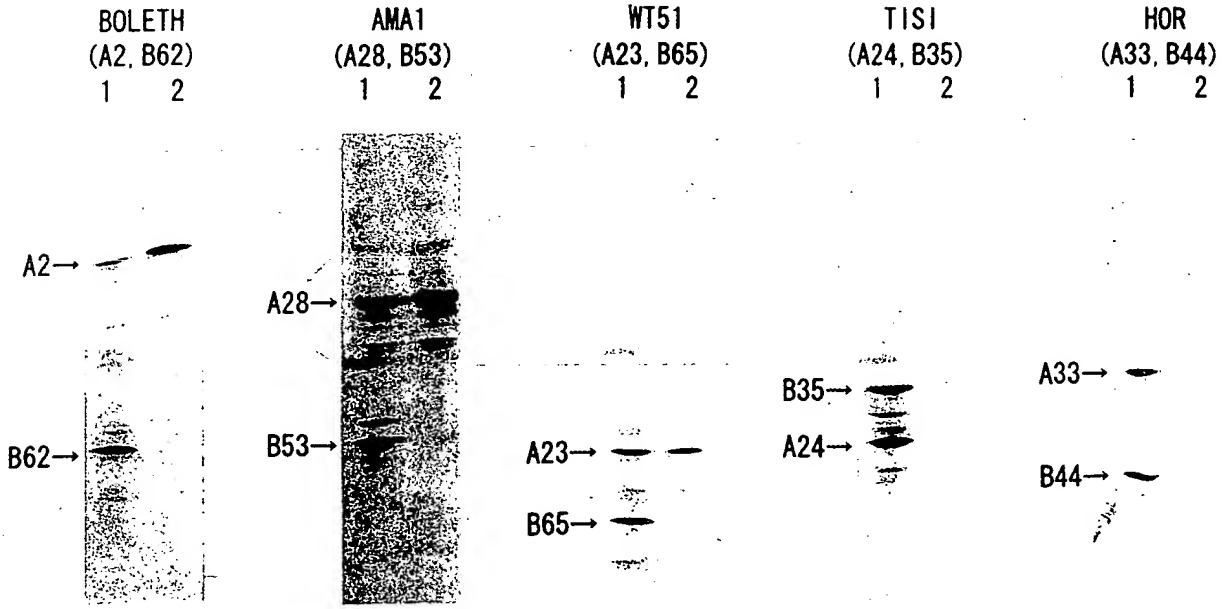

Figure 2. IEF pattern of lysates from homozygous B-lymphoblastoid cell lines immunoprecipitated with either the monomorphic class I mAb W6/32 (lane 1) or 1-145 (lane2). HOR (HLA-A33) was used as a negative control. Immunoprecipitation and one dimensional isoelectric focusing (IEF) gel analysis were performed as described (5).

	111									120							127 ↓			130									140
Consensus	R	G	Y	H	Q	D	A	Y	D	G	K	D	Y	I	A	L	N	E	D	L	R	S	W	T	A	A	D	M	A	A
A*0201	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-
A*6802	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-
A*6901	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-
A*2301	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-
A*2402	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-
A*0101	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A*0301	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A*1101	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	150									160										170
	Q	I	T	K	R	K	W	E	A	A	H	V	A	E	Q	L	R	A	Y	L	E	G	T	C	V	E	W	L	R	R
A*0201	-	T	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A*6802	-	T	-	-	H	-	-	-	-	-	-	-	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A*6901	-	T	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A*2301	-	-	-	Q	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	G	-	-	-
A*2402	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	D	G	-	-	-
A*0101	-	-	-	-	-	-	-	-	-	V	-	A	-	-	-	R	-	V	-	-	-	-	R	-	-	D	G	-	-	-
A*0301	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-
A*1101	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	Q	-	-	-	-	-	-	R	-	-	-	-	-	-	-

Figure 3. Comparison of the deduced amino acid sequences of the portion of the alpha 2 domain of HLA-A alleles. The standard one-letter amino acid code is used and the identity with consensus is shown by a dash. Amino acids at position 127 are shared by HLA-A2.-A28.-A23 and -A24.

Table 2.
Absorption Test

Absorbed Cells	Target Cells				
	RML (A2,B51)	WT51 (A23,B65)	AMA1 (A28,B53)	TISI (A24,B35)	HOM-2 (A3,B27)
RML (A2,B51)	0	0	0	0	0
WT51(A23,B65)	x2	0	x4	0	0
AMA1(A28,B53)	0	0	0	0	0
TISI(A24,B35)	x64	x32	x32	0	0
HOM-2(A3,B27)	x128	x256	x256	x2	0
Pre-absorption	x800	x800	x400	x4	0

The titers were determined by PA assay. The supernatant was diluted 20 times in RPMI 1640 and 0.08ml was absorbed with $3x10^7$ B cell lines. The number of cells necessary for absorption had been determined by the serial titration of the cells. "0" indicates no reactivity.

A210, -A68,-A69 and -A23. However, no cytotoxic reactivity was observed against 111 panel cells expressing HLA-A24. No reactivity was observed among 63 panel cells without HLA-A2, -A210, -A68, -A69, -A23, either. Thus, in microcytotoxicity assay using PBLs, 1-145 reacted with HLA-A2, -A28 and -A23 expressing cells but not with HLA-A24 expressing cells. This property of 1-145 was useful in distinguishing HLA-A23 antigens from HLA-A24 antigens in routine laboratory tissue typing.

In order to confirm the specificity of 1-145 mAb, absorption study was carried out (Table 2). Overnight incubation of 1-145 supernatant with HLA-A3 expressing HOM-2 showed a little nonspecific absorption of the antibody, which resulted in the two to eight fold reduction of the titer. On the other hand, absorption of the supernatant with either RML(HLA-A2), WT51(HLA-A23) or AMA1(HLA-A28) left little or no reactivity against these cell lines. As expected, HLA-A24 expressing TISI had a marginal absorption capacity.

The reactivity of 1-145 mAb was also confirmed biochemically by immunoprecipitating metabolically labeled EBV-transformed B cell lysates with the antibody. IEF banding pattern showed that 1-145 precipitated HLA-A2, -A28 and -A23 antigens but did not precipitate HLA-A24 antigen (Fig. 2). It is conceivable that the reaction of 1-145 to HLA-A24 antigen is too weak to be detected by our current immunoprecipitation protocol.

Comparison of the published amino acid sequence data suggests that Lys at position 127 may be critical for 1-145 binding (Fig.3). This is in agreement with the previous report that proposed Lys at position 127 as the HLA-A2-28-9 epitope (6). The higher affinity against HLA-A2 and -A28 also suggest Thr-142 and His-145 may important in stabilizing the binding. These amino acid residues has been proposed as A2-28 epitope (6). The deduced amino acid sequence of HLA-A23 (A*2301) shows only three amino acid difference with that of HLA-A24 (A*2402) in the extra cellular domains. HLA-A24 antigen has Lys-144, His-151 and Gln-156 whereas HLA-A23 antigen has Gln-144, Arg-151 and Leu-156 (Fig.3). Thus, the lower affinity of 1-145 mAb reactivity against HLA-A24 compared to HLA-A23 may result from the conformational change induced by any of the three amino acids substitutions at positions 144, 151 and 156 on the alpha 2 domain. Further analysis of 1-145 binding using site-directed mutagenesis technique would be necessary to clarify these assumption.

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Third Keystone Symposium on Cellular Immunology and the Immunotherapy of Cancer

Tumor Antigens Recognized by T Cells

In Vitro Induction of Specific Cytotoxic T Lymphocytes Using Recombinant Single-Chain MHC Class I/Peptide Complexes

Yu-Chun Lone, Iris Motta, Estelle Mottez, Yannik Guilloux, Annick Lim, Frédéric Demay, Jean-Pierre Levraud, Philippe Kourilsky, and Jean-Pierre Abastado

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Summary: We have previously described the production and purification of a murine single-chain, soluble recombinant major histocompatibility complex (MHC) class I molecule (SC-Kd). A similar strategy was devised to produce a recombinant HLA-A2.1 (SC-A2) molecule. The latter was composed of the first three domains of the HLA-A2.1 heavy chain connected to human β_2-microglobulin through a spacer of 15 amino acids. Immunoaffinity-purified SC-A2 molecules were correctly folded and biologically functional. They specifically bound HLA-A2-restricted peptides and induced a peptide-specific cytotoxic T lymphocyte (CTL) clone to proliferate and secrete interleukin-2. The ability of murine and human SC-MHC molecules to elicit primary CTLs in vitro was next investigated. When coated in high density onto beads, complexes of antigenic peptide and SC-Kd or SC-A2 molecules efficiently induced a specific primary CTL response in vitro. Furthermore, the structural features of these CTLs were characterized by T cell receptor-β chain analysis, which revealed rearrangements very similar, if not identical, to those found in CTLs generated by in vivo immunization. Such single-chain, soluble recombinant MHC class I molecules should provide a useful tool in particular for peptide binding assays and for in vitro primary CTL induction to identify immunogenic peptides such as those derived from known tumor-associated antigens. **Key Words:** Major histocompatibility complex—Cytotoxic T lymphocytes—Clonal expansion.

Major histocompatibility complex (MHC) class I molecules present antigen to $CD8^+$ T cell receptor (TCR) αβ T cells in the form of a 8- to 10-amino-acid-long peptide bound into their groove. These peptides usually contain within their sequence two or three anchor residues that interact with corresponding binding pockets in the MHC molecule (1–4). Definition of specific MHC motifs allows prediction of those peptides derived from viral or tumor antigens potentially immunogenic for cytotoxic T lymphocytes (CTLs). Such peptides will often bind with a high affinity to the relevant MHC molecules (5). However, it has been reported in several instances that peptides that do not fit the consensus motif nevertheless bind to MHC molecules and are immunogenic (6). The availability of single-chain recombinant MHC class I molecules would, among other uses, make it possible to develop a rapid and sensitive assay for the identification of T cell epitopes of potential interest.

We have previously described the production and purification of a murine single-chain, soluble recombinant MHC class I molecule (SC-Kd) (7,8). This molecule can

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be obtained in large quantities from transfected CHO cells. It can be loaded homogeneously with synthetic peptides and was shown to select the same peptide repertoire as the native cell surface-associated H-2K^d (9,10). Such MHC/peptide complexes when dimerized by monoclonal antibodies (mAbs) can specifically induce interleukin (IL) 2 secretion by the cognate T cells (11).

These findings have prompted us to develop a similar strategy for the production of a single-chain recombinant HLA-A2.1 molecule. An SC-A2 construct was engineered by connecting the first three domains of HLA-A2.1 heavy chain to human β_2-microglobulin (β_2M) through a 15-amino-acid-long spacer and transfected into rat basophil cells (RBL-2H3). The SC-A2 molecule was secreted into the culture medium from which it was purified. In the present study, we show that the purified SC-A2 molecules are correctly folded and bind specifically HLA-A2-restricted antigenic peptides but not murine H-2K^b- or H-2K^d-restricted peptides. Moreover, SC-A2/peptide complexes specifically induced a proliferative response of cognate CTLs, indicating that the SC-A2 molecules are biologically active.

In recent years, various approaches have been considered in attempts to develop in vitro protocols for the induction of cytotoxic T cell responses against viral and tumor antigens (12–21). One major requirement appears to be a high density of peptide/MHC complexes on the surface of the antigen-presenting cells (APCs). Bearing this in mind, we also addressed the question of the effectiveness of complexes of recombinant MHC class I molecules and peptides in the elicitation of specific CTLs, using, in a human system, the SC-A2 and, in a murine system, the SC-Kd molecules. The results presented in this article demonstrate that such molecules, when coated onto beads, are highly efficient in presenting bound peptides for the induction of peptide-specific CTLs. Importantly, when the TCR-β repertoire of these CTLs was analyzed using the sensitive Immunoscope technique (23,23), we found significant expansions of cell populations bearing the same BV, BJ, and CDR3 length as those observed in in vivo immunizations, implying the generation of qualitatively similar CTLs in vivo and in vitro.

MATERIALS AND METHODS

Peptides and Serological Reagents

Peptides used in this study are specified in Table 1. They were purchased from Neosystem (Strasbourg, France). The anti-HLA-ABC mAb W6/32 and antihuman β_2M mAb FMC 16 were purchased from Valbiotech (Paris, France). The antihuman β_2M mAb B2.62.2 (24) and the anti-HLA-A2.1 mAbs BB7.2, MA2.1, PA2.1, and 5H7 were previously described (25–28).

Cell Lines

The rat basophil cell line RBL-2H3 has been previously described (29) and was a kind gift from Dr. R. Klausner. The processing-defective 174CEM.T2 cell line (13), P815 cells (TIB 64, American Type Culture Collection, Rockville, MD, U.S.A.), and P815/HLA-CW3 transfectants (30) were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 10 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 5×10^{-5} *M* 2-mercaptoethanol, and antibiotics (complete FCS medium).

Plasmid Construct and Basophil Cell Transfection

The human β_2M sequence (31) was amplified by polymerase chain reaction (PCR) using the following primers: GGGGGGATCCATCCAGCGTACTCCAAAGATT and GGGGAAGCTTCCAATCCAAATGCGGCATCTT. After digestion with *Bam*HI and *Hin*dIII, the 350-bp amplified fragment was cloned into M13mp18, yielding M13/huβ_2M. The HLA-A2.1 sequence was derived from a full-length cDNA kindly provided by P. Parham (32). First, a 410-bp fragment produced by digestion with *Sal*I and *Kpn*I and coding for the first 113 residues of HLA-A2 was purified. Then, a 500-bp fragment coding for residues 114–274 from HLA-A2.1 was amplified by PCR and fused to one $(Gly)_4Ser$ motif using the following primers: CTTCCTCCGCGGGTACCACC and GGAAGGATCCACCGCCACCCCATCTCAGGGTGAGGGGCTTGGGCAA. This PCR product was digested by *Kpn*I and *Bam*HI. The two fragments were fused together and cloned into M13mp19 cleaved with *Sal*I and *Bam*HI, yielding M13-α1α2α3. The *Sal*I–*Bam*HI 920-bp fragment of M13-α1α2α3 encoding resi-

TABLE 1. *Synthetic peptides used*

Peptide	Sequence	Restriction	Ref.
F10V	FLPSDYFPSV	HLA-A2	33
I9V	ILKEPVHGV	HLA-A2	60
Melan-A/MART-1	AAGIGILTV	HLA-A2	45
MAGE-3	FLWGPRALV	HLA-A2	61
NA17-A	VLPDVFIRCV	HLA-A2	35
M58-66	GILGFVFTL	HLA-A2	43
CW3	RYLKNGKETL	H-2K^d	30
HA	IYSTVASSL	H-2K^d	62
NP	TYQRTRALV	H-2K^d	41

dues 1–274 of HLA-A2 fused to a flexible 5-residue-long linker was then inserted into M13/huβ_2M cleaved by *Bam*HI and *Sal*I, yielding M13-SC-A2 (5). Ten additional residues Ser(Gly)$_4$Ser(Gly)$_4$ were subsequently inserted into the linker by cloning the following oligonucleotides; GATCAGGCGGTGGTGGGTCGGGTGGCGGCG and GATCCGCCGCCACCCGACCCACCACCGCCT, at the *Bam*HI site of M13-SC-A2 (5). The resulting phage, called M13-SC-A2 (15) was sequenced. The *Hin*dIII 1,300-bp fragment containing the complete insert was then subcloned into pRc/CMV (Invitrogen, Leek, The Netherlands) at the *Hin*dIII site, yielding pCMV.SC-A2. The transcription orientation under the CMV promoter was first checked by restriction map and nucleotide sequencing. RBL-2H3 cells (10^7) were transfected with 10 μg of *Pvu*I-linearized pCMV.SC-A2 plasmid DNA, using a Cellject electroporation system (Eurogentec, Sart Tilman, Belgium) set at single pulse, and 250 V with a shunt resistance of 192 Ω and a capacitance of 450 μF. After 24 h, cells were selected in complete FCS culture medium containing 1 mg/ml of G418 (Gibco, Gaithersburg, MD, U.S.A.). Further selection was obtained by a gradual increase of the dose of antibiotic to 4 mg/ml.

Preparation of Soluble SC-42

SC-A2 was purified from the supernatant of pCMV.SC-A2-transfected RBL-2H3 cells by affinity chromatography using the mAb W6/32.

Surface Plasmon Resonance

Analyses were conducted on a BIAcore (Pharmacia Biosensor) at 20°C using a flow rate of 5 μl/min. The anti-HLA-A2 mAb BB7.2 was immobilized on a CM5 sensor chip previously activated with a 35-μl pulse of NHS/EDC [N-hydroxysuccinimide/N-ethyl-N′-(3 dimethylaminopropyl) carbodiimide hydrochloride] mixture, and unreacted moieties were blocked with ethanolamine 1 *M* (pH 8.5).

Peptide Binding Assay

Peptide binding to SC-A2 was assayed by competition using radiolabeled peptide F10V (33). This peptide was labeled to a specific activity of 43 mCi/mmol using chloramine T-catalyzed iodination as described previously (34). SC-A2 (7 μg) was incubated for 2 h at room temperature with 4.5 μ*M* labeled F10V in the presence of the indicated concentration of the competitor peptide. Unbound peptide was eliminated by ultrafiltration using Microcon 30 (Amicon, Beverly, MA, U.S.A.) and extensive washing with phosphate-buffered saline. Radioactivity in the retentate was measured in a gamma spectrometer (Gammamatic; Kontron).

Proliferation and IL-2 Secretion

The NA17-221 CTL clone, specific for the NA17-A peptide presented by HLA-A2.1 (35), was cultured in 96-well plaques in 200 μl of complete FCS medium in the presence of the indicated concentration of peptide/SC-A2 complexes dimerized through the FMC16 mAb. Proliferation was assayed after 48 h by incorporation of [^{3}H]thymidine. IL-2 secretion was monitored at 24 h by measuring the capacity of culture supernatant to sustain the proliferation of the IL-2-dependent cell line CTLL-2.

In Vitro CTL Induction

Male DBA/2 mice were obtained from the Centre d'Elevage R. Janvier (Le Genest-Saint Isle, France) and were used at 8–12 weeks of age. Dendritic cells (DCs) were prepared, according to standard protocols of enrichment, through centrifugation of spleen cells on a 35% bovine serum albumin solution (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) and plastic adherence of the low-density spleen cell fraction, as described in ref. 36. Nylon wool-enriched T cells (1.5×10^7) were cultured either with 10^5 DCs pulsed 4–6 h previously with 100 μg CW3 or NP peptide or with SC-Kd/peptide complexes coupled onto beads (5×10^6). Cultures were performed in 5 ml of complete FCS medium supplemented with 20 ng/ml mouse recombinant (r) IL-6 (Immugenex Corp., Los Angeles, CA, U.S.A.) and 0.5 ng/ml mouse rIL-12 (R&D Systems Europe Ltd., U.K.) in 25-cm^2 flasks standing upright at 37°C in a humidified atmosphere containing 6% CO_2. Three and 5 days after culture initiation, human rIL-2 (Immugenex) was added to the cultures to yield a final concentration of 10 U/ml. Cultures were restimulated on day 7 using peptide (50 μg)-pulsed irradiated (3,000 rads) spleen cells (1×10^7) in 2 ml complete FCS medium containing 1 ng/ml mouse rIL-7 (Immugenex) and 10 U/ml human rIL-2. Additional human rIL-2 (20 U/ml final) was provided on day 10 and the cultures tested for cytolytic activity on day 12–14.

In vitro CTL responses were elicited in peripheral blood mononuclear cells (PBMCs) from HLA-A2.1 normal healthy volunteers, using SC-A2/peptide complexes coupled onto beads. Briefly, Ficoll-purified PBMCs (3×10^7) were cultured with SC-A2/peptide complexes coupled onto beads (10^7). The cultures were maintained in complete RPMI-1640 supplemented with 10% heat-inactivated AB human serum (complete AB medium), and rIL-7 (10 ng/ml; Genzyme S.A., Paris, France), and

rIL-12 (2 ng/ml; Genzyme) at 37°C in a 5% CO_2 incubator. The culture medium with cytokines was replenished every 3 days. At day 10, the cultures were restimulated with 10^7 irradiated (3,000 rads) peptide-pulsed Con A-activated autologous T blasts or with 140 m*M* of monomeric SC-A2/peptide complexes. The cultures were maintained in complete AB medium containing human rIL-7 (10 ng/ml) and rIL-2 (10 U/ml). Fresh medium with cytokines was added every 3 days. Cytotoxic activity was measured 8 days following restimulation.

Cytotoxicity Assay

P815 cells ($3–5 \times 10^6$) pulsed with 50 μg CW3 or NP peptide and P815/HLA-CW3 transfectants were radiolabeled with ^{51}Cr for 90 min at 37°C in RPMI-1640 medium. They were then washed three times in the same medium, resuspended in complete FCS medium, and then added (5,000) to effector cells. The T2 cell line (3×10^6 cells) was pulsed overnight with 200 μg of peptide in 2 ml of RPMI-1640 medium without serum at 26°C. The cells were then radiolabeled with ^{51}Cr as indicated for the murine cell lines. Cytolytic activity was determined in a standard 4-h ^{51}Cr release assay using U-bottom 96-well plates. Percent cytotoxicity was determined from the following formula: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined by lysis of target cells with 1% Triton X-100 (Sigma). Spontaneous ^{51}Cr release values varied between 10 and 20% of total incorporated label.

Repertoire Analysis

Human and mouse TCR-β analyses were performed as previously described (23,37). Briefly, total RNA was extracted and subjected to reverse transcription and PCR amplification using TCR-BC- and TCR-BV-specific primers. PCR products were labeled with a nested fluorescent TCR-BC-specific primer and size fractionated in a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Analyses were then carried out using the Immunoscope software.

RESULTS

Structural Characterization of Single-Chain MHC Class I Molecules

The mouse SC-Kd molecule has been extensively characterized (9–11). The production of single-chain human HLA-A2 molecules was undertaken using a similar approach. An SC-A2 construct was engineered, comprising the signal sequence and the first three domains of HLA-A2.1 heavy chain cDNA fused to the coding sequence of human β_2M through a 15-amino-acid flexible linker. This construct was transfected into RBL-2H3 cells. Stable transfectants were selected and screened by PCR for the integrated gene construct. Positive clones were analyzed for SC-A2 production. One clone yielded ~500 μg of SC-A2/L culture supernatant and was selected for further studies.

The secreted SC-A2 was purified by immunoaffinity chromatography using the mAb W6/32 (anti-HLA-A.B.C) and characterized by surface plasmon resonance using the mAbs BB7.2 (anti-HLA-A2, -A69) and B2.62.2 (antihuman β_2M). Results shown in Fig. 1 demonstrate that it displays both HLA-A2- and human β_2M-derived serological determinants. That the recombinant SC-A2 is correctly folded was verified by enzyme-linked immunoadsorbent assay performed with a panel of mAbs comprising W6/32, BB7.2, 5H7 (anti-3rd domain of HLA-A2), PA2.1 (anti-HLA-A2), B2.62.2, and BBM.1 (antihuman β_2M). All mAbs tested reacted alone or in sandwich (anti-HLA-A2/antihuman β_2M) with the recombinant molecule (data not shown), suggesting that this latter displays the correct conformation.

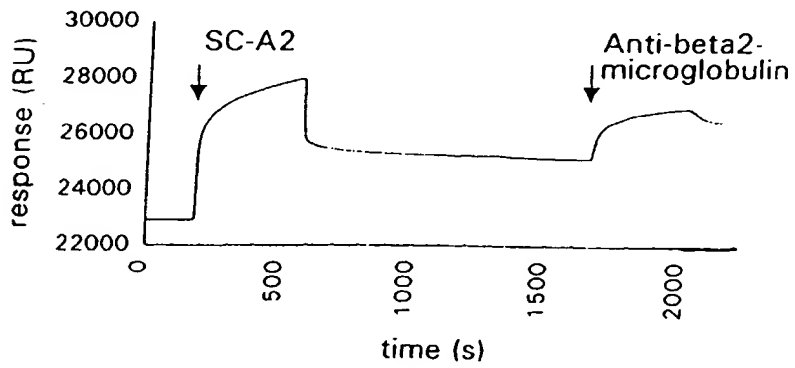

FIG. 1. Detection of SC-A2 molecule by surface plasmon resonance. The anti-HLA-A2 mAb BB7.2 was immobilized on a CM5 sensor chip. The antihuman β_2M mAb (B2.62.2) did not bind to this sensor chip. SC-A2 preparation was injected at 200 s. After 400 s, the surface was washed for another 1,000 s. Then the B2.62.2 mAb was injected for 400 s. See text for abbreviations.

Functional Characterization of the Single-Chain MHC Class I M lecules

Two functional tests, which had previously been used to characterize the murine SC-Kd molecule, were performed to assess the properties of the SC-A2 molecule (11,38). We first verified that SC-A2 specifically binds HLA-A2-restricted peptides. A synthetic peptide, F10V, derived from hepatitis B virus and known to bind to HLA-A2 (33) was used as standard for competition experiments. Radiolabeled F10V peptide was mixed with SC-A2, as described in Materials and Methods. Unbound peptide was removed by ultrafiltration and the radioactivity associated with SC-A2 was measured. A significant binding to SC-A2 was observed (Fig. 2A). From the specific activity of the radiolabeled peptide, we calculated that >50% of the SC-A2 protein was active in peptide binding. This binding was peptide specific since it was inhibited in a dose-dependent manner by unlabeled HLA-A2-restricted peptides (F10V, I9V, M58-66, MAGE-3, NA17-A, Melan-A/MART-1) but not by H-2K^d-restricted peptides (CW3, HA) added in excess as competitors (Fig. 2B). These results indicate that the recombinant SC-A2 molecule binds synthetic peptides and displays the same specificity as the native cell surface-associated HLA-A2 molecule.

The ability of recombinant SC-A2 to act as an antigen-presenting molecule was next evaluated. Purified SC-A2 was loaded with the NA17-A melanoma peptide (35), dimerized using an anti-β_2M mAb, and used to stimulate the NA17-221 CTL clone. In both proliferation (Fig. 3A) and IL-2 secretion (Fig. 3B) assays, the response obtained was peptide specific since SC-A2 loaded with an unrelated peptide (MAGE-3) had no activity. As already reported (39), the NA17-A peptide alone had no effect on the NA17-221 CTL clone (data not shown). The specific responses observed with the NA17-221 clone demonstrate that the SC-A2/peptide complex interacts productively with its cognate TCR.

Taken together, these data establish that the SC-A2 molecule displays all anticipated properties and shares the same features as its murine counterpart. We therefore undertook a series of parallel experiments to assess the ability of these molecules to elicit specific CTL responses in vitro.

Coupling of Recombinant MHC/Peptide Complexes to Beads

We were interested in determining whether specific CTLs could be induced in vitro by SC-MHC molecules loaded with an antigenic peptide. To maximize the density of SC-MHC/peptide complexes, we prepared beads to which large numbers of such complexes were bound through an antibody specific for either the α-3 domain of SC-Kd or the β_2M domain of SC-A2.

To evaluate the coupling efficiency of SC-A2 onto beads, we performed the following experiment. Radiolabeled F10V peptide was incubated with purified SC-A2. Unbound peptide was removed by ultrafiltration, and the radioactive MHC/peptide complex was incubated with tosylactivated Dynabeads coupled with an antihuman β_2M antibody. After extensive washing, radioactivity as-

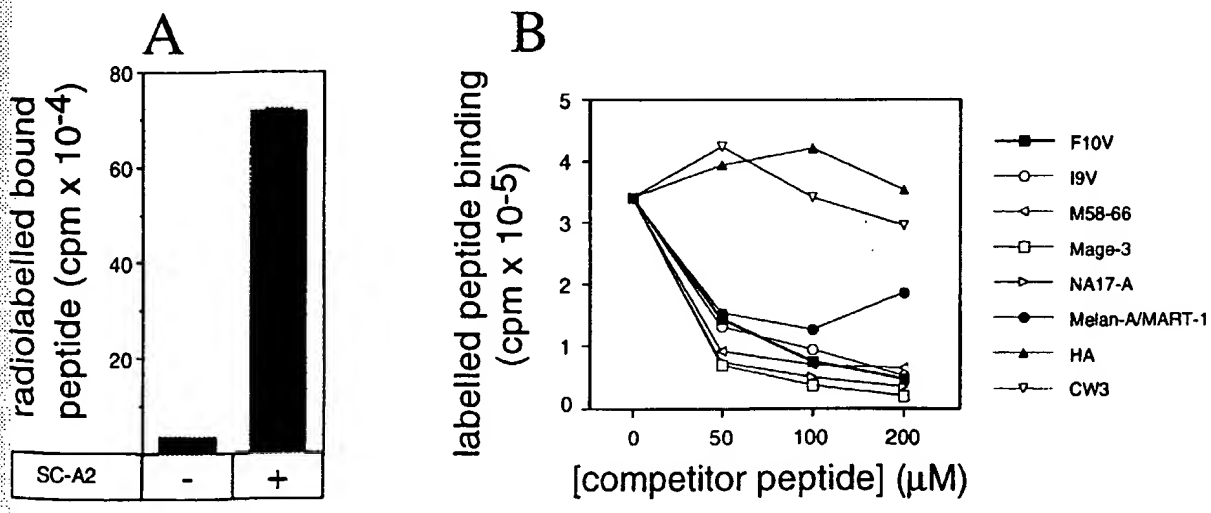

FIG. 2. Specificity of HLA-A2.1-restricted peptide binding assay. **A:** Binding of radiolabeled F10V peptide to SC-A2 molecules. **B:** Competitive inhibition of the binding of peptides to SC-A2 molecules. The HLA-A2.1-restricted peptides (F10V, I9V, M58-66, MAGE-3, NA17-A, and Melan-A/MART-1) show significant inhibition of the binding of labeled peptide, whereas the H-2K^d-restricted peptides (CW3, HA) do not.

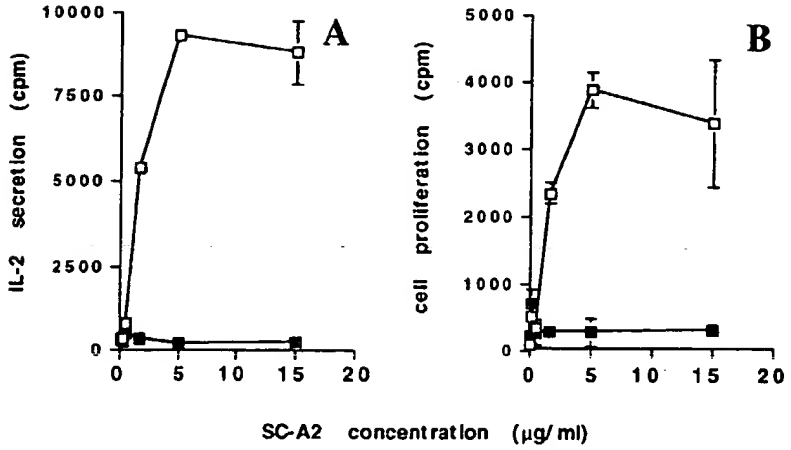

FIG. 3. Direct incorporation of [^{3}H]thymidine or IL-2 secretion by CTL NA17 221, showing the proliferative response of the CTLs to appropriately restricted peptide. The NA17-221 CTL clone specific for the NA17-A peptide presented by HLA-A2.1 was cultured with the indicated concentration of dimerized peptide/SC-A2. (Open squares). NA17-A peptide; (filled squares), MAGE-3 peptide. A: IL-2 content was assayed at 24 h by CTLL-2 proliferation. B: T cell proliferation was determined at 48 h at [^{3}H]thymidine incorporation. See text for abbreviations.

sociated with the beads was measured and used to evaluate the quantity of SC-A2 coupled to the beads. In the absence of SC-A2, 100-fold less radioactivity was found associated with the beads. We calculated that 10^5/SC-A2 molecules were coupled on each bead. This number corresponds to a density of 1.7×10^{15} molecules/m^2, which is at least 10 times greater than the total density of MHC molecules at the surface of a natural APC. Furthermore, considering that a natural APC presents in the order of 10^4 different peptides and that the maximal representation of a given peptide at the surface is 10% (40), we concluded that the density of a specific MHC/peptide complex at the surface of the beads is at least 2 orders of magnitude greater than on a natural APC.

In Vitro Induction of Specific CTLs by MHC/Peptide Complexes Coupled to Beads

The ability of the beads to stimulate CTLs in vitro was then evaluated in mouse and human systems. SC-Kd was loaded with the K^d-restricted CW3(170–179) peptide derived from HLA-CW3 (30) or the NP(147–155) peptide derived from the nucleoprotein of the influenza virus (41). Such SC-Kd/peptide complexes were then coupled onto beads, as indicated above, and added to nylon wool-nonadherent splenocytes of DBA/2 mice. For comparison as positive control, stimulation was also performed with peptide-pulsed DCs as previously described (16,20, 42). Seven days later, all cultures were restimulated with irradiated CW3 or NP peptide-pulsed spleen cells. Five to 6 days later, cytotoxic activity was measured on P815 and P815/HLA-CW3 transfectants or on CW3 and NP peptide-pulsed P815 target cells. As shown in Fig. 4, significant peptide-specific lytic activity was detected whether SC-Kd/peptide-loaded beads or DCs were used as the immunizing stimulus. Cytotoxicity curves suggested that both procedures were quite comparable in the efficiency of induction and expansion of peptide-specific CTLs.

A parallel study was conducted in humans, using peripheral blood lymphocytes (PBLs) from naive HLA-A2 donors. Such PBLs were cultured for 10 days with beads coupled with complexes of SC-A2/influenza A matrix protein M58-66 peptide (43,44). The cultures were restimulated once with either M58-66 peptide-pulsed Con A blasts or SC-A2/M58-66 peptide complexes for 8 days and then tested for cytotoxic activity on peptide-loaded T2 target cells. A significant specific lysis was observed (Fig. 5). We next extended this observation using a second HLA-A2-restricted peptide derived from the Melan-A/MART-1 antigen (45,46) (data not shown).

Taken together, these results suggest that MHC/peptide complexes coupled on beads are about as efficient to induce primary CTLs in vitro as professional APCs such as DCs.

Display of TCR Rearrangements by In Vitro Induced CTLs Similar to That of CTLs Primed In Vivo

The above data show that MHC/peptide complexes coupled on beads do generate CTLs in vitro in the absence of added co-stimulatory molecules. Of importance, however, is the assessment of the relevance of such CTLs with respect to those elicited as a consequence of in vivo immunization. To settle this point, we took advantage of the observation that, in some instances, T cell responses to given antigens display public rearrangements, i.e., rearrangements that emerge recurrently upon immunization of distinct individuals (22,47). Public TCR-BV rearrangements in the response against the K^d-restricted CW3 peptide are particularly well documented

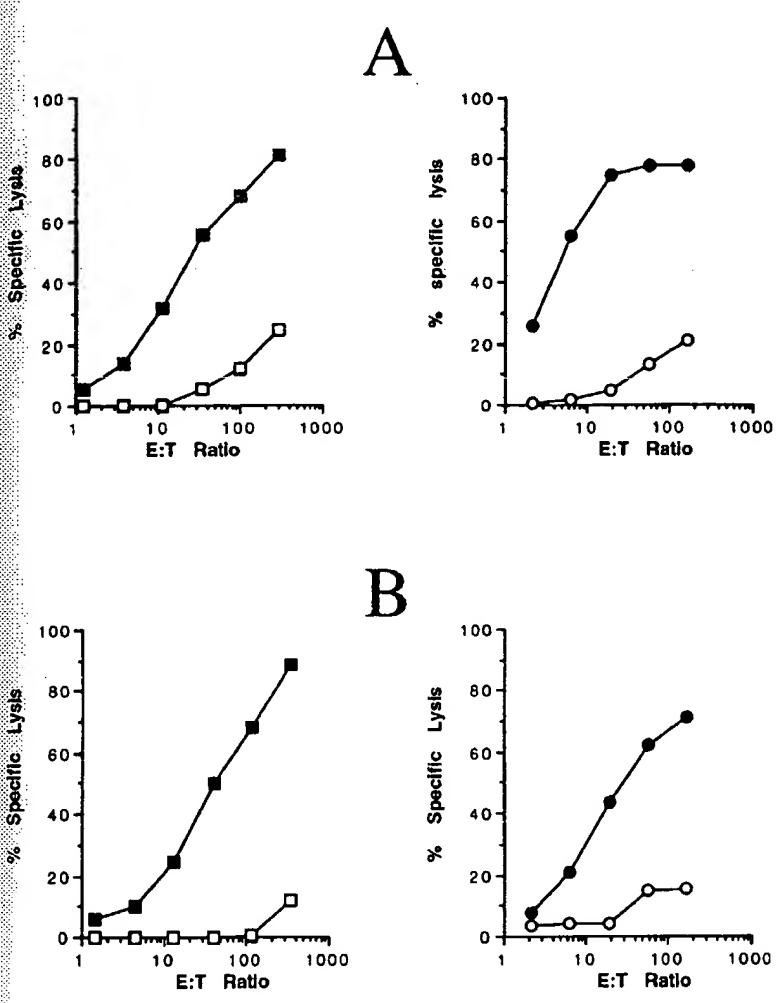

FIG. 4. Induction of specific CTLs by SC-Kd/peptide complexes coupled to beads or DCs. CTLs were induced by peptide-pulsed DCs (A) or SC-Kd/peptide complexes coupled to beads (B). Peptides used for the induction were CW3 (left) and NP (right). Cytotoxic activity was measured on P815 (open squares), P815-CW3 (filled squares), P815 pulsed with NP (filled circles), or CW3 (open circles). See text for abbreviations.

(48–51). In humans, a public response against the HLA-A2-restricted peptide derived from the influenza virus A matrix protein M58-66 has also been reported (52,53). We therefore further compared the in vitro with the in vivo CTL responses to these two peptides using our previously described Immunoscope technology (22,23).

The TCR repertoire of CTL populations isolated from DBA/2 mice immunized with P815-HLA-CW3 transfectants reflects the selective expansion of BV10-bearing lymphocytes. Moreover, upon analysis of 23 CW3-specific clones, all BV10$^+$, only five BJ segments were found, the BJ1S2 being predominant, and all displayed CDR3 loops of six amino acids in length (48). Profiles obtained for the TCR-BV10 of DBA/2 spleen cell populations stimulated in vitro by the CW3 peptide are represented in Fig. 6. As can be seen, the CW3 peptide presented either by SC-Kd beads or DCs leads to the generation of T cell populations that exhibit, for the TCR-BV10, a major peak corresponding to a six-amino-acid-long CDR3. Further analysis shows a predominant association of such CDR3 with a BJ1S2 segment (data not shown). This TCR-BV10 repertoire thus displays the same features as those of the CW3-reactive CTLs generated by in vivo priming. For comparison, Fig. 6 shows the TCR-BV10 profile obtained for T cells generated against the NP peptide in a parallel experiment.

It has been reported that in most HLA-A2.1 subjects, an M58-66 peptide-specific CTL response was detectable subsequent to influenza A infection. Interestingly, M58-66 peptide-specific CTL lines derived from 21 unrelated HLA-A2 individuals show TCR-BV17 as the dominant BV segment used. Expansion of CD8$^+$ BV17$^+$ cells correlated with M58-66 peptide-specific lysis (52,53). Moreover, sequence analysis of 38 M58-66 peptide-specific BV17 transcripts from 13 subjects revealed extensive conservation in the eight-amino-acid long CDR3 region. The TCR-BV17 profiles obtained from control unstimulated PBLs and PBLs stimulated with

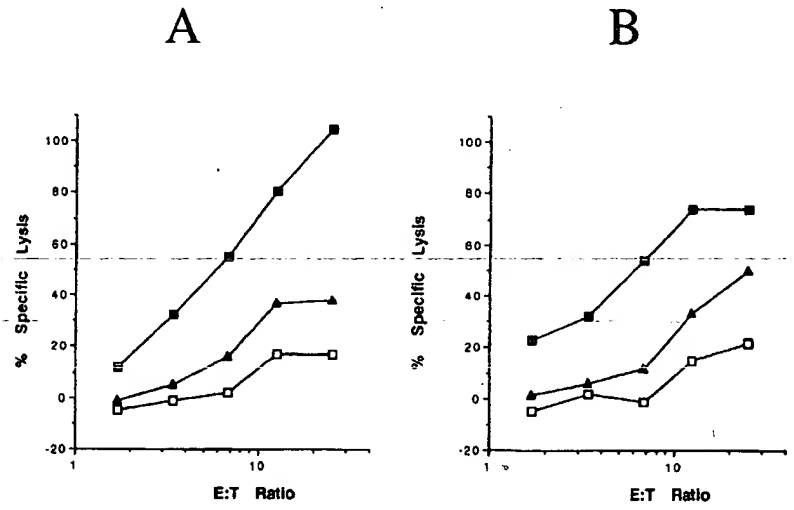

FIG. 5. Induction of specific cytotoxic T lymphocytes (CTLs) by SC-A2/peptide complexes coupled to beads. The CTL cultures induced with SC-A2/M58-66 complexes coupled to beads were restimulated with peptide-pulsed Con A-activated blasts (A) or 140 m*M* monomeric SC-A2/M58-66 (B). Cytotoxic activity was assayed on T2 cells (open squares), M58-66-pulsed T2 cells (filled squares), or MAGE-3-pulsed T2 cells (filled triangles).

beads coated with SC-A2/M58-66 peptide complexes are presented in Fig. 7. The TCR-BV17 profiles of stimulated PBLs exhibit a major peak corresponding to a CDR3 of eight amino acids in length (Fig. 7A and B), suggesting a specific expansion of that T cell population that had been implicated in M58-66 peptide-specific lysis. In contrast, control PBLs displayed a Gaussian-like TCR-BV17 profile (Fig. 7C), indicative of a polyclonal repertoire. For comparison, the TCR-BV2 profiles are also presented and display a Gaussian-like shape in control and stimulated PBLs.

These results indicate that in vitro induction protocols using beads can expand T cell populations with structural characteristics similar to those generated by in vivo immunization.

DISCUSSION

Given the increased importance recognized for CTLs in the control of long-lasting diseases, including chronic viral diseases (e.g., AIDS) and cancers, we have looked for means of activating specific CTLs in vitro. We report

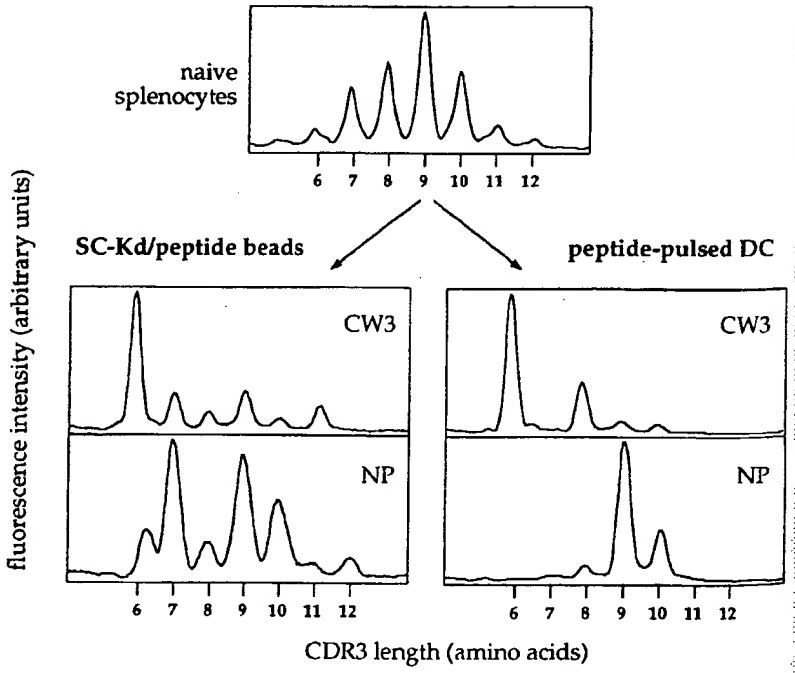

FIG. 6. TCR-BV10 repertoire of CTLs induced with SC-Kd/CW3 complexes coupled to beads and with CW3-pulsed DCs. CTLs were induced as described in the legend to Fig. 4. Total RNA was extracted and cDNA amplified using BV10- and BC-specific primers. After labeling with a nested fluorescent BC-specific primer, the size distribution of the product was resolved in a denaturing acrylamide gel in an automated sequencer. Size in amino acids of the CDR3 is indicated as defined by Chothia et al. (63). A major peak at six amino acids is the hallmark of CW3-specific CTLs. RNA extracted from naive splenocytes or from NP-specific CTLs is shown for comparison. See text for abbreviations.

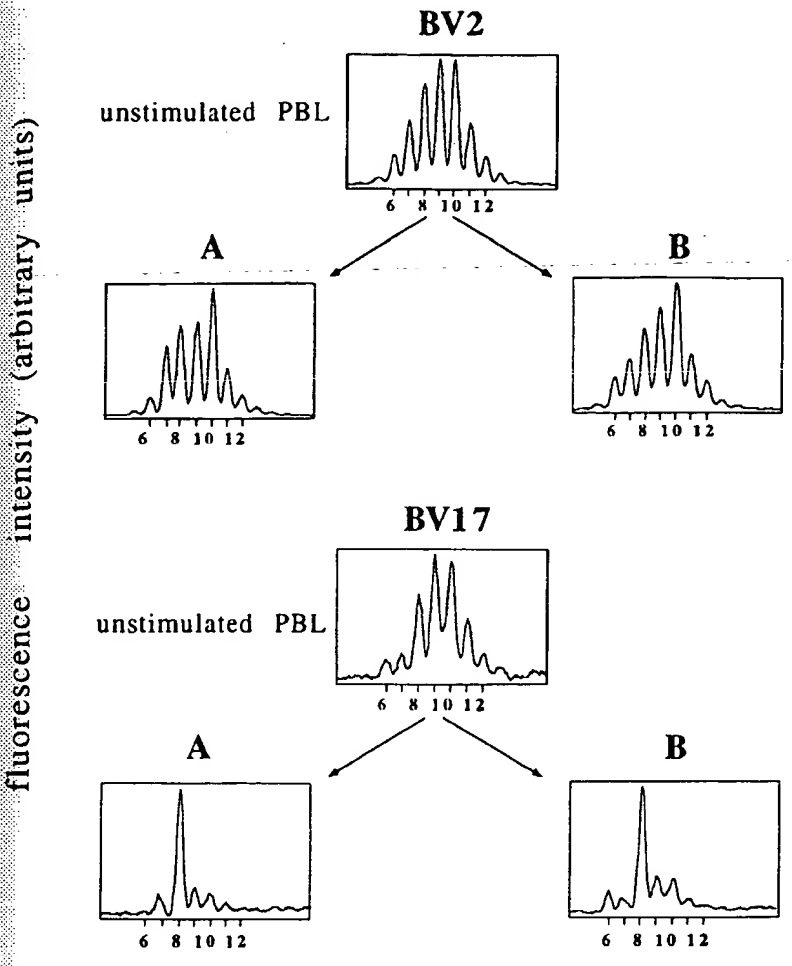

FIG. 7. TCR-BV17 and -BV2 repertoires of CTLs induced with SC-A2/M58-66 coupled on beads. CTLs generated with SC-A2/M58-66 coupled on beads were restimulated with peptide-pulsed Con A blasts [A] or monomeric SC-A2/M58-66 complexes [B] as in Fig. 5. Total RNA was analyzed using BV17- **(bottom)** or BV2- **(top)** specific primers. The major peak observed with BV17 primers corresponding to a CDR3 of eight amino acids in length is the hallmark of M58-66-specific CTLs. The BV2 repertoires and those of unstimulated PBLs are shown for comparison. See text for abbreviations.

here that recombinant single-chain MHC molecules coupled at high density onto beads can efficiently activate primary CTLs and that, when the comparison was possible, these CTLs display similar characteristics to those produced by in vivo immunization.

MHC class I molecules are heterodimers made of a polymorphic trans-membrane heavy chain not covalently associated with β_2M. β_2M exchange takes place at the cell surface in tissue culture so that up to 25% of the heavy chain can associate with heterologous β_2M (54). Moreover, in the absence of β_2M, heavy chains are usually unstable and collapse. We resolved this problem by engineering a murine SC-Kd molecule (8). Using a similar strategy, two groups recently reported the construction of a single-chain, membrane-associated HLA-A2.1 (55,56). In the present study, we have produced and purified a recombinant soluble single-chain HLA-A2.1 molecule (SC-A2). We first showed that this purified SC-A2 molecule is correctly folded and specifically binds HLA-A2-restricted antigenic peptides, suggesting that it selects the same peptide repertoire as the native cell surface-associated HLA-A2 molecule. Moreover, SC-A2/peptide complexes stimulate peptide-specific CTLs to proliferate and secrete IL-2, indicating that the SC-A2 molecule interacts with the appropriate TCR. Thus, with regard to both structure and function, the SC-A2 molecule displays features similar to those described for its murine counterpart, SC-Kd (8–11).

We next tested the ability of SC-Kd and SC-A2/peptide complexes to activate peptide-specific CTL precursors in vitro. We show that HLA-CW3 and influenza virus NP peptide-specific CTL expansion can indeed be induced in naive mouse spleen cell cultures stimulated with SC-Kd/peptide complexes coupled onto beads. Ef-

ficacy of these latter is comparable with that of known professional APCs such as DCs. With regard to the HLA-CW3 peptide, the CTLs generated by either mode of antigen presentation were able to lyse P815/HLA-CW3 transfectants, indicating the recognition by these CTLs of endogenously processed epitopes. This observation is important since it has been reported in some instances that CTLs generated in vitro failed to kill targets expressing naturally processed antigen (57,58), and recognition of endogenously expressed antigen is regarded as an indicator of in vivo relevance (59). TCR-β repertoire analysis of T cell populations stimulated in vitro by CW3 peptide-pulsed DC or SC-Kd/CW3 peptide provides evidence for the significant and specific expansion of $BV10^+$ lymphocytes with a CDR3 of six amino acids in length associated with a predominant BJ1S2 segment. CTLs generated from in vivo immunization with HLA-CW3 bear the same characteristics. Similarly, an influenza A M58-66 peptide- and a Melan-A/MART1-specific CTL response were elicited in PBL from HLA-A2.1 healthy donors, using SC-A2/peptide-coupled beads as the immunizing stimulus. These induced CTLs efficiently kill peptide-pulsed T2 cells. In the case of the influenza-specific CTLs, where a public response had been described (53), analysis of their TCR-BV repertoire indicates the specific expansion of cell populations using predominantly TCR-BV17 with an eight-amino-acid-long CDR3 region, as described for M58-66 peptide-specific CTLs generated after in vivo influenza virus infection.

The availability of SC-MHC class I molecules is of interest since these molecules can be used not only in a rapid, sensitive, and reliable MHC/peptide binding assay to identify high-affinity MHC binding T cell epitopes but also in in vitro primary CTL induction to define, among the MHC binders, those peptides that are immunogenic. The present report constitutes the first demonstration that biologically active recombinant murine and human MHC class I molecules may be used for primary peptide-specific CTL in vitro induction and that the CTLs generated are qualitatively similar to those elicited in vivo. Such a system may be helpful in detailed analyses of human or murine CTL repertoire and in unraveling the mechanisms of CTL priming. It may also be considered for the in vitro production of specific CTL effector cells for clinical use. We are currently engineering additional single-chain HLA molecules that would allow coping with human MHC polymorphism.

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TRANSPLANTATION

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THE USE OF MAGNETIC BEADS COATED WITH SOLUBLE HLA CLASS I OR CLASS II PROTEINS IN ANTIBODY SCREENING AND FOR SPECIFICITY DETERMINATION OF DONOR–REACTIVE ANTIBODIES[1]

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An adequate method that will permit rapid specificity determination of donor-reactive antibodies is urgently needed. Such a method could also be used for monitoring the presence of HLA antibodies in panel screening. We here describe a method using magnetic b ads coated with soluble HLA antigens that can be directly added to patient serum for efficient absorption of HLA antibodies. The entire procedure takes 45 min, and can therefore be easily adopted for use in acute crossmatching situations. Furthermore, since no live cells are required for identification of alloantibodies in the screening for HLA specific panel-reactive antibodies, it can be used as an ideal complement for screening of panel-reactive antibodies. Binding of antibodies to the antigen-coated beads is easily visualized using flow cytometry. A new method for quick purification of soluble HLA antigens from a pool of lymphocytes or thrombocytes is also presented.

Over the last couple of years the importance of the humoral presensitization status of transplant recipients has been increasingly recognized. With greater understanding of the precise specificity of the pretransplant antibody status of recipients and careful recipient selection in cadaver donor kidney transplantation, it is clear that graft survival will improve.

We, and others, have found that there exists an association between the presence of low levels of B cell-reactive HLA specific antibodies, detected in the microlymphocytotoxicity test, or the presence of non-complement-binding antibodies detected only in the flow cytometric crossmatch and early acute rejection episodes following allogeneic kidney transplantation (*1–3*). Since many patients have non-HLA-specific antibodies that may cause false-positive reactions, especially in the lymphocytotoxic crossmatch (*3, 4*), we have devised methods that will allow quick HLA specificity determination of donor-reactive cytotoxic antibodies based on the absorption by soluble HLA class I and/or class II antigens (*5*). This has been of special importance, since a weakly positive B cell crossmatch has no predictive value as such. When specificity tests are included, the presence of HLA- specific B cell–reactive antibodies has a high predictive value and correlates with a high incidence of early acute rejection episodes (*1*).

There is a definite need for specificity assessment also of panel-reactive antibodies in patients on the waiting list for kidney, liver, heart, heart/lung, or pancreas or islet cell transplantation. The importance of donor-reactive HLA-specific alloantibodies has been established for kidney and liver transplants, whereas few data are available regarding other organs (*6, 7*).

As yet, only few tests that will permit the determination of HLA specificity of lymphocytotoxic or donor cell-binding antibodies are available (*5*). Recently, the ELISA test (Prastat, Baxter) for screening of panel-reactive antibodies has become available, but the cost for HLA antibody class I specificity determination in a single sample was initially stated to be 100 pounds. Furthermore, the test is not useful in a crossmatch situation.

Our earlier specificity test was based on the blocking of positive crossmatch reactions using soluble HLA antigens (*5*). In the present study, we demonstrate that the test is facilitated even more if soluble antigens are absorbed onto paramagnetic microbeads. Since such beads can be tested also in flow cytometry, this further development will allow testing for antibody specificity, not only for crossmatching purposes, but also for the screening of panel-reactive antibodies. A new method for the quick purification of soluble HLA antigens from a panel of lymphocytes is also presented, based on the absorption of solubilized HLA class I and class II antigens by antibodies against HLA class I or class II antigens adsorbed onto magnetic microbeads.

MATERIALS AND METHODS

Antibodies. Specific immunoadsorbents were prepared using commercially available goat antimouse immunoglobulin-coated Mini-Macs paramagnetic beads. Mouse monoclonal antibodies directed to a nonpolymorphic determinant on either HLA class I or II molecules (W6/32, Serotec, and BU25, The Binding Site, respectively) were linked to the goat antimouse antibody-coated magnetic beads.

Other monoclonal antibodies used were anti-CD3, -CD4, (T cell markers) -CD19 (B cell marker), and -CD45 (panleukocyte marker) (Becton Dickinson).

Pooled sera from allosensitized patients already characterized by us as containing HLA alloantibodies were used as positive controls (*5*). Sera from healthy nontransfused AB blood group males served as negative controls.

Preparation of cell lysates. Human thrombocytes from 80–100 individuals and spleen lymphocytes from 20 individuals (10×10^8) pooled separately were the sources for the extraction of HLA class I and II antigens. The cells were solubilized using lysis buffer (10 mM Tris, 0.5% Non-idet P40, 0.2 mM phenyl methylsulfonyl fluoride, 0.02% sodium azide, and 0.15 M sodium chloride, pH 7.2) at 4°C for

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1-hr. Insoluble cell debris was removed by centrifugation at 10,000 [mult]g for 30 min at 4°C.

Preclearing using MiniMacs paramagnetic beads. Two preclearing steps were used to reduce nonspecific binding of proteins in the lysate to the final product of beads coated with soluble HLA antigens.

For this purpose 10 ml of crude lysate was mixed at 4°C for 1 hr with 50 μl of goat antimouse antibody-coated MiniMacs paramagnetic beads. The beads were separated using a magnet. Next, the lysate was incubated with 500 μl sheep antimouse IgG antibody-coated Dyna beads (Dynal, Norway) coupled with mouse IgG (1 mg/ml) (Sigma, St. Louis, MO). After incubation for 1 hr, the beads were separated using a magnet and the supernatant formed the precleared lysate.

Binding of mouse antihuman HLA class I/II monoclonal antibodies to the magnetic beads. Goat antimouse Ig–coated MiniMacs beads (50 μl) were linked with 50 μl of either mouse antihuman HLA class I (1 mg/ml) (1:50) or HLA class II (0.5 mg/ml) (1:40) monoclonal antibodies for 30 min at 4°C. The beads were then washed three times with phosphate-buffered saline (PBS) supplemented with 5 mM EDTA and 0.5% bovine serum albumin using a magnet.

Polyacrylamide gel electrophoresis with Phast gel. In order to test the purity of the HLA class I and class II antigens bound to the beads, the fractions were tested using gel electrophoresis after elution from the beads. For this purpose, the primary mouse monoclonal antibodies were initially crosslinked with the goat antimouse antibodies coated on the beads using the reagent dimethylpimelimidate (DMP) in order to prevent elution of the primary antibodies along with the antigens. Then 100 μl of the beads was first washed with 0.2 M triethanolamine, pH 9.0, and 5 ml of 20 mM of DMP was added to the beads and incubated for 45 min at room temperature. The beads were washed with 5 ml of 0.2 M ethanolamine, pH 8.0, and incubated with the same solution for 2 hr at room temperature. Thereafter the beads were washed twice with PBS. The beads were resuspended in 50 μl of sample buffer (0.0625 M Tris, 10% glycerol, 2.3% SDS and bromophenol blue at pH 6.8) and heated at 100°C for 5–6 min. The samples were placed against a strong magnet and 10 μl of the supernatant added to the gel sample wells. Pharmacia-LKB (Biotechnology, Uppsala, Sweden)–prefabricated small (4 × 5-cm) polyacrylamide gradient gels (10–15%) were run, with sample loads as small as 50 ng/lane, and electroblotted (semidry) or silver-stained, all in a PhastSystem.

Binding of soluble HLA antigens present in the lysate to the HLA class I or class II antibody–coated magnetic beads. The HLA class I or class II antibody-coated magnetic beads were incubated with the precleared lysate for one hr at 4°C. The beads were then separated using a magnet and washed three times with the abovementioned buffer. The washed beads were resuspended in 500 μl of the same buffer and kept stored at 4°C until further use.

Specific absorption assay for HLA specificity determination of antibodies in patient's serum. All patient and control sera were first incubated with sheep antimouse Ig-coated Dynabeads coupled with mouse IgG, to remove all nonspecific binding. This was done by incubating 200 μl of patient's or control serum with 20 μl of the abovementioned beads at 4°C for 15 min on a rock and roller (Swelab, Sweden). The beads were separated using a magnet.

Packed HLA class I or class II antigen-coated magnetic beads (5 μl) were incubated with 25 μl of precleared negative/positive control/patient's serum at 4°C for 20 min. The beads were separated using a magnet and washed three times using PBS-EDTA-BSA buffer. Then 5 μl of 1:4 diluted fluoresceinated $F(ab)'_2$ fragments of either anti-IgG or anti-IgM antibodies (Southern Biotechnology, Birmingham) was added and incubated in the dark at 4°C for 10 min. The beads were washed twice with the abovementioned buffer and resuspended in 200–300 μl of the same buffer. The beads were then ready to be analyzed in a flow cytometer.

Analysis. A fluorescence-activated cell analyzer (FACStrak, Becton Dickinson, Mountain View, CA) with an argon laser producing 400 mW of light at 488 nm was used for all analyses. Data were collected with logarithmic amplification, and fluorescence intensity was displyed on a 256-channel, 4-decade log scale delinated in arbitrary log units. Fluorescence signals from 5000 beads were counted and the percentage of FITC-positive cells was recorded. Histograms of beads versus log fluoresence intensity were generated. A shift in the mean fluorescence of ≥ 20 channels in the test sample as compared with the negative control was considered positive.

In addition, we studied retrospectively 50 consecutive crossmatch sera (current sera) for donor-specific antibodies using donor T and B spleen lymphocytes. The crossmatch procedures used were the microcytotoxicity (*1*) and the flow cytometric (*10*) assays. The HLA specificity and immunoglobulin class(es) of the antibodies were determined.

RESULTS

Prior to the absorption of antibodies in the patient's serum, the proteins bound to the magnetic beads were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The amount of eluted proteins from the HLA class I antibody–coated beads (100 μl) was 0.8 mg while that from the class II antibody–coated beads (100 μl) was 0.3 mg. Lanes 1 and 2 in Figure 1 show the results of using anti–class I and anti–class II monoclonal antibody–coated magnetic beads to absorb their respective antigens. Figure 1 shows bands corresponding to chains of 44 kDa (heavy chain of class I), 34 kDa, and 28 kDa (alpha and beta chains of class II).

Ability of HLA class I and class II antigen–coated magnetic beads to specifically bind monoclonal and allogeneic HLA-specific antibodies. The HLA class I and class II antigen–coated magnetic beads were first tested for the ability to bind specifically to their respective monoclonal antibodies. Figure 2, A and B shows that the HLA class I and class II antigen–coated beads specifically bound their respective monoclonal antibodies.

As seen in Figure 3, A and B, no binding of HLA class I monoclonal antibodies to HLA class II antigen–coated beads or of HLA class II monoclonal antibodies to HLA class I antigen–coated beads was observed. In addition, no nonspecific binding of anti-CD3, -CD4, -CD19, and -CD45 to the HLA antigen–coated beads was observed (Fig. 4, A and B).

The HLA antigen -coated beads were then tested for their ability to bind alloantibodies in sera from broadly sensitized patients known to have HLA antibodies (tested by platelet

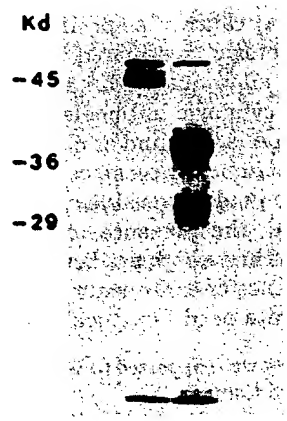

FIGURE 1. Elution of HLA class I (lane 1) and class II antigens (lane 2) from HLA class I and class II–coated paramagnetic microbeads used as immunoadsorbents.

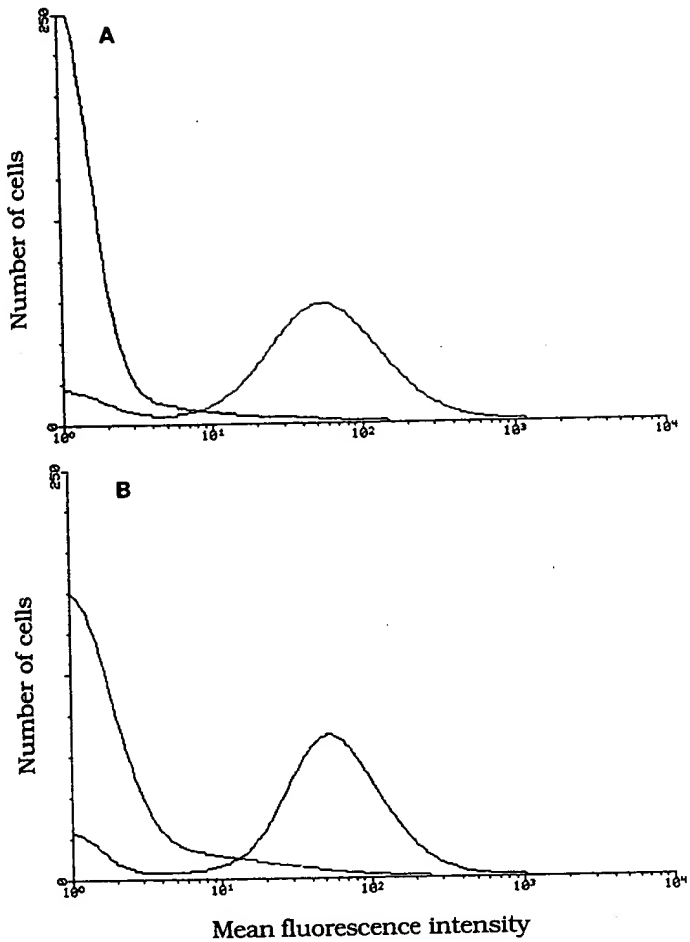

FIGURE 2. Binding of mouse antihuman monoclonal antibodies to HLA class I (A) or class II (B) to microbeads coated with HLA class I and class II antigens, respectively. AB serum was used as the negative control.

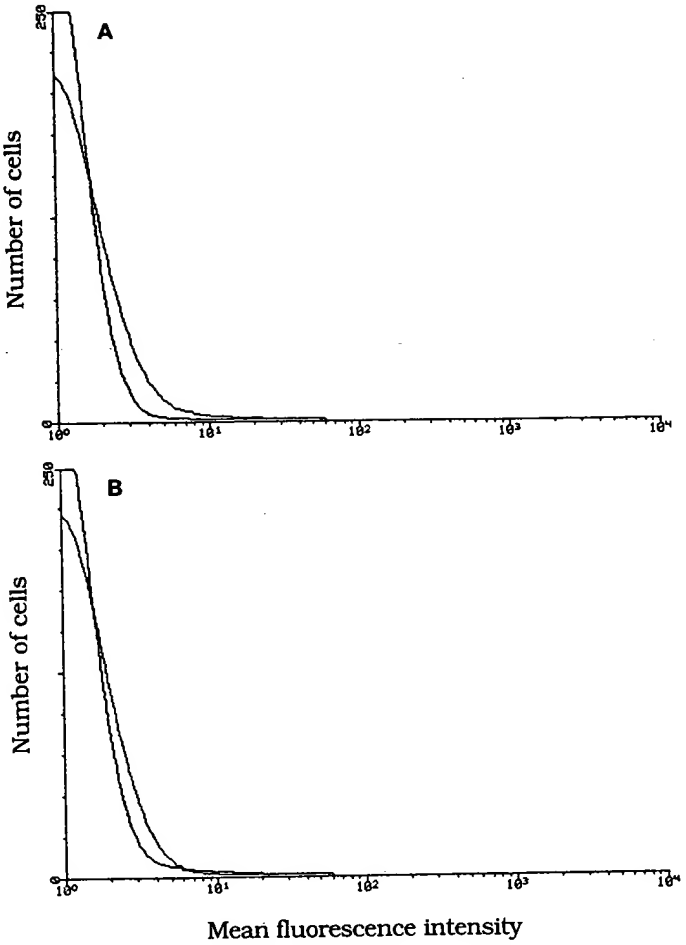

FIGURE 3. No binding of HLA class II antihuman monoclonal antibodies to microbeads coated with HLA class I antigens (A) or of HLA class I antihuman monoclonal antibodies to microbeads coated with HLA class II antigens (B) was observed. AB serum was used as the negative control.

absorptions and/or in a blocking assay) (*5*). Figure 5, A and B shows the binding of HLA class I or class II antigen–coated beads to their respective alloantibodies. In addition, to test whether the method was sensitive and specific enough to detect low levels of HLA antibodies of a single specificity, we used the beads to absorb HLA antibodies from the sera of two patients known to have low cytotoxic levels (1:2) of antibodies to either HLA-A2 or HLA-DR4. The results are shown in Figure 6, A and B. Both sera gave positive reactions with a clear increase in the mean fluorescence channel as compared with the negative control.

In Figure 7, A and B, a few examples of sera (some of which contained HLA antibodies, and some of which did not) reacting with the HLA antigen-coated beads from patients awaiting a kidney transplant are shown.

In addition, Table 1 shows the comparison of the inhibition assay as described in our earlier study (*5*), with the current method. Concordant results were obtained in every case using both methods.

Definition of HLA–reactive antibodies and their immunoglobulin class specificity as detected in crossmatch sera of renal transplant patients. Of 50 patients, 13 lost their grafts, and 12/50 had early acute rejections (lost within one month after transplantation). Twenty of these 50 had donor-specific antibodies (Table 2), and 4 of those 20 had weakly positive (15%-25%) B cell cytotoxic antibodies, while 16 had positive flow cytometric crossmatches. Four of the 20 had non-HLA antibodies only, while 16 had HLA class I or class II, or both types of antibodies. The presence of HLA-reactive antibodies correlated significantly with early acute rejection and early graft loss ($P < 0.001$).

DISCUSSION

Recently, ample evidence supporting the importance of characterization of antibodies in the crossmatch sera of patients awaiting an organ transplant in selecting an HLA class I crossmatch–negative donor has been described (*2*, *4*). The currently available techniques for specificity determinations are not reliable in distinguishing HLA-reactive antibodies from the non-HLA ones in a serum with mixtures of antibodies and cannot be done acutely in a cadaver donor situation (*5*).

The method described here entails the use of soluble HLA antigen-coated paramagnetic beads that are added to a patient's serum, and the bound HLA alloantibodies can thereafter be detected by fluoresceinated secondary antibodies. The entire procedure takes 45 min. The method is simple,

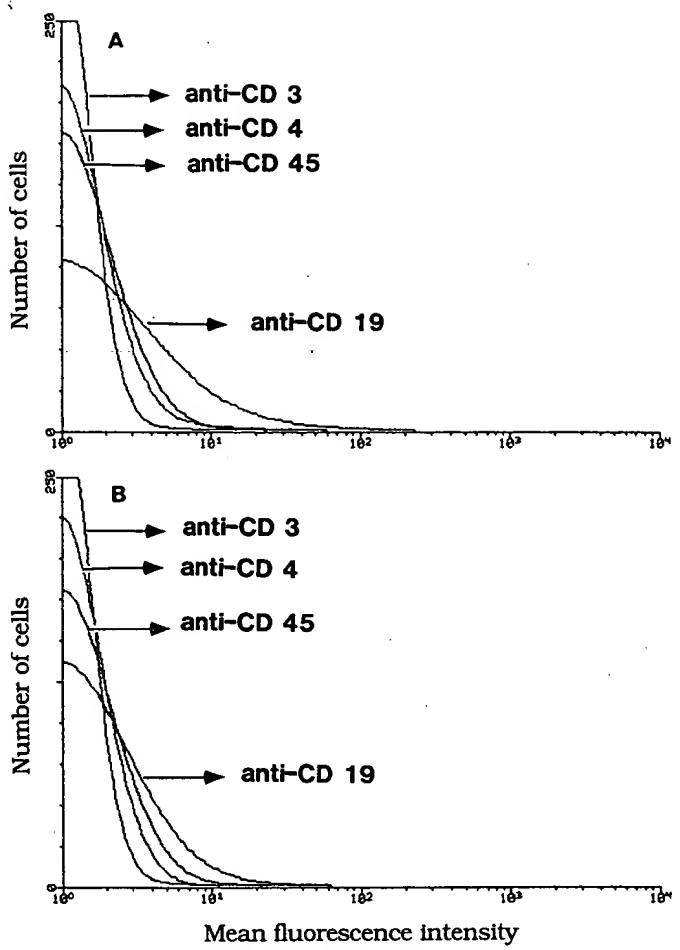

FIGURE 4. No binding of control monoclonal antibodies to -CD3, -CD4, -CD19, and -CD45 to microbeads coated with HLA class I (a) or class II antigens (B) was observed.

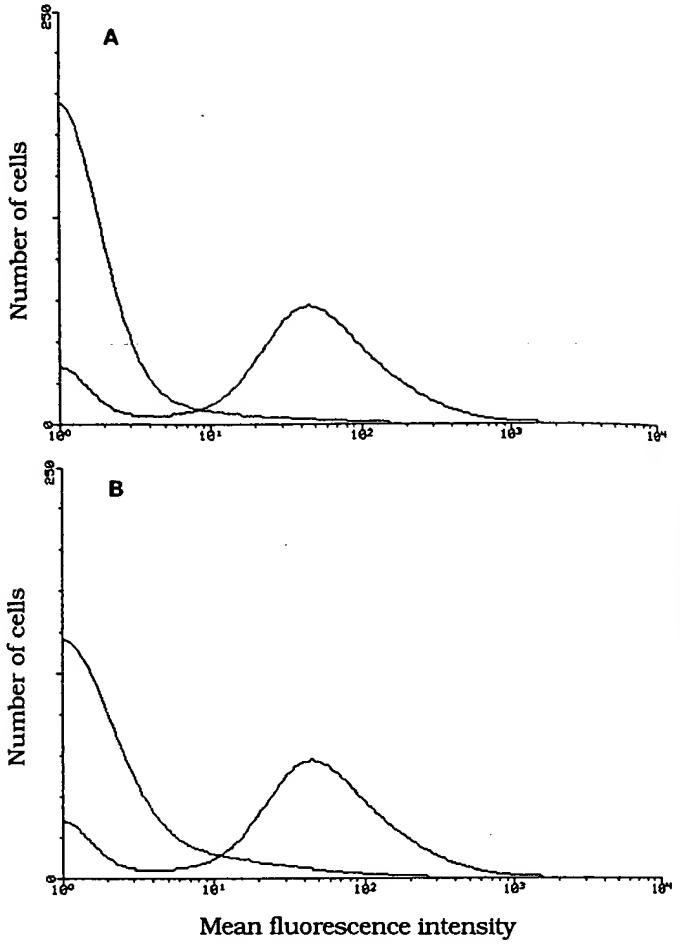

FIGURE 5. Binding of HLA class I (A) or class II (B) antibdies from a pool of broadly allosensitized patients to microbeads coated with HLA class I and class II antigens, respectively. AB serum was used as the negative control.

reliable, specific, and rapid in determining HLA antibodies in a patient's serum.

Earlier (*5*), we described the use of soluble HLA antigens to specifically absorb HLA antibodies in a patient's serum. Although that method is reliable and simple, it has two major disadvantages (1) time consumption and the tedious method for extraction of the soluble HLA antigens, and (2) the possible unreliability of the method in detecting the presence of HLA-reactive antibodies in a serum containing mixtures of antibodies. However, using this method, both these problems are solved. The immunoprecipitation of the HLA antigens using the MiniMacs beads was as effective as the affinity antibody-coated Sepharose immunoadsorbent method (*5*), but the time required was much shorter. In addition, less nonspecific binding of proteins in the lysate to the paramagnetic beads was observed as compared with the Sepharose columns, resulting in fewer interference bands and a lower background (results not shown). It is advisable to use smaller aliquots of lysate during the preclearing step and the step involving binding of the HLA antigens in the lysate to the beads. This increases the probability of an effective contact. In addition, it is important that all materials such as cells and solutions used be kept cold (4°C). Higher temperatures and prolonged incubation times may lead to increased nonspecific binding (*8*). Since analysis of the direct binding of HLA antibodies in a patient's serum to the paramagnetic beads can be done, the test is ideal for use in direct flow cytometry.

This method can therefore be used to detect HLA antibodies in the monthly screening of the sera of patients awaiting an organ transplant. The objective of an HLA antibody screening/identification for transplantation is, first, to detect all HLA antibodies that might be potentially important clinically, and second, to use this information for patient selection before donor crossmatching. To accomplish this it is necessary to use a panel of lymphocytes to reliably gauge whether the patient is "sensitized" or not— specifically to obtain as much serologic knowledge as possible about the HLA antibody specificity. Once this is established (*9*), it may be tedious and probably not feasible to continue further quarterly panel screening using lymphocytes. In that case monitoring of the detected HLA antibodies in a patient's serum may be performed using the HLA antigen-coated paramagnetic beads. This method avoids the problem of cytotoxicity caused by autoantibodies and other antibodies giving false-positive results. Lastly, it is essential that the HLA antibody screening method be specific, convenient, and sensitive, and that the method should not necessarily depend directly on a functional attribute of the antibody (such as complement

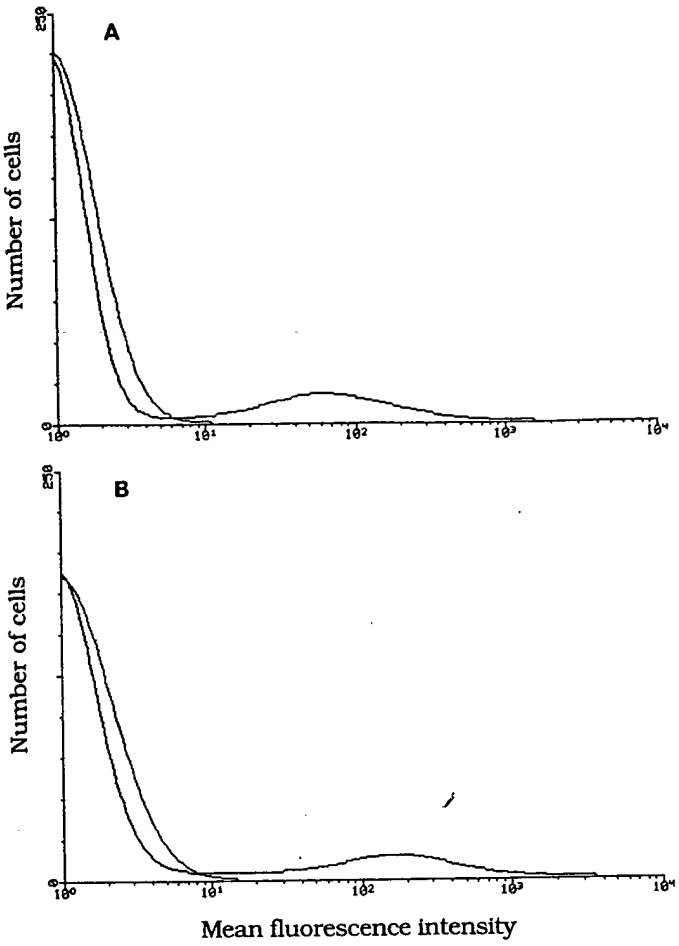

FIGURE 6. Binding of low-titered HLA-A2 antibodies from a patient's serum to microbeads coated with HLA class I antigens (A) and DR4 antibodies from another patient's serum to microbeads coated with HLA class II antigens. (B) AB serum was used as the negative control.

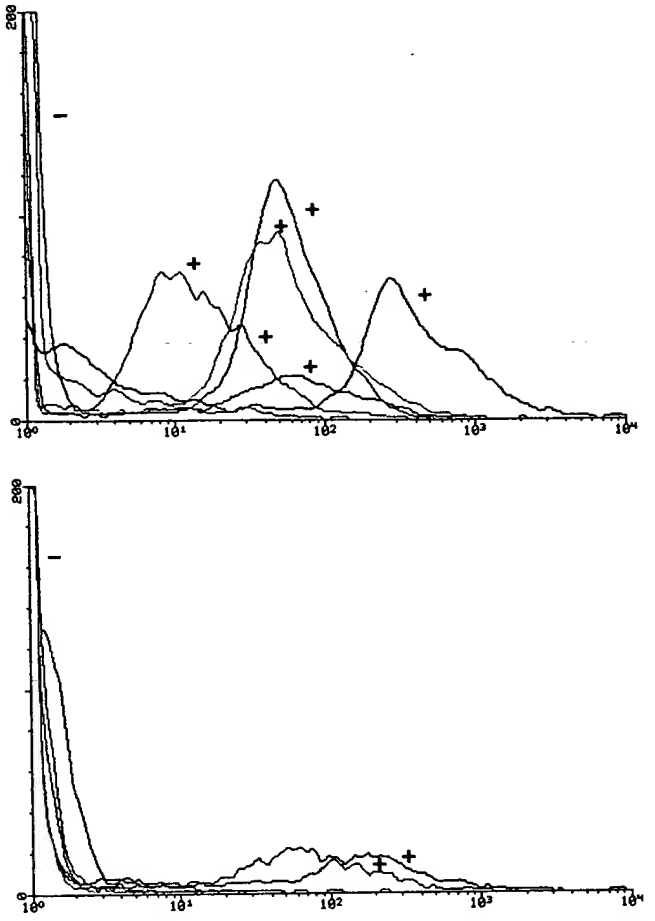

FIGURE 7. A few examples of sera—some containing HLA-reactive antibodies (+) and some not (-)—reacted with beads coated with HLA class I (A) or class II (B) antigens.

fixation) for its detection, since non-complement-fixing HLA antibodies may also be deleterious directly or indirectly to the allograft (*10*). Since no living cells are required for identification of alloantibodies, the present method can be used as an efficient, time and cost–effective supplement to the HLA antibody screening method.

This method may also be used for detection of donor-specific HLA antibodies. HLA antibodies can be detected by reacting the patient's serum with donor lymphocytes before and after absorption with the beads. However, once again the limitation of the method in such a case would be the detection of HLA alloantibodies in a mixture of high-titered non-HLA and low-titered HLA-reactive antibodies, as discussed in our earlier study (*6*). Once again, since the HLA antigen preparations are from a pool of cells from different persons, the likelihood of missing the rarer HLA antigens remains. However, we have used this method satisfactorily to detect donor-specific antibodies in sera from twenty patients with mixtures of HLA and non-HLA antibodies (Table 2).

This method is especially cost-effective, since the paramagnetic beads can be recycled. This is achieved by cross-linking the goat antimouse antibody-coated beads with the primary antihuman HLA class I/or II monoclonal antibodies using the reagent dimethylpimelimidate (11). In this way, elution of the antigen-alloantibody complex by a suitable buffer will not elute the primary antibody, and the beads may be reused for further analyses. In addition, as little as 5 μl of the beads is required for analysis of one serum sample. A total of 250 tests may be performed with HLA antigens extracted from 80–100 outdated platelet samples.

The advantage of using the MiniMacs paramagnetic microbeads is the very small size of these particles (diameter < 0.5 μm); the binding reaction; that the beads do not aggregate; and, more important, that they have no autofluorescence, which will allow analysis in the flow cytometer (*8*). However, the one major disadvantage of these paramagnetic beads is that for efficient separation, a strong magnetic field is required, usually obtained using a coated stainless-steel wire column placed temporarily in the field of a powerful permanent magnet. Therefore, for extensive separations and washings this would imply a cost limitation, since these columns are not reusable. To overcome this problem we have used an ordinary magnet (Dynal) with an incubation time of 10 min while using it for separation, which has worked satisfactorily.

We believe that our method is adequate for the detection of HLA alloantibodies in a patient's serum, and it combines simplicity, efficiency, and specificity. We believe that com-

TABLE 1. Comparison of the inhibition assay using soluble HLA, antigens with the absorption assay using soluble HLA antigen–coated microbeads for the detection of HLA antibodies in patients' sera

Pts.[a]	Titers	Inhibition with soluble HLA class I antigens	Inhibition with soluble HLA class II antigens	Absorption with HLA class I–coated beads	Absorption with HLA class II–coated beads	Deduced specificity	Ig class
1.	1:500	Yes	No	Yes	No	Class I	IgG
2.	1:50	No	No	No	No	Non-HLA	IgM
3.	1:100	Yes	Yes	Yes	Yes	Class I and class II	IgG + IgM
5.	1:20	No	No	No	No	Non-HLA	IgM
7.	1:50	Yes	Yes	Yes	Yes	Class I and class II	IgG + IgM
9.	1:100	Yes	No	Yes	No	Class I	IgG
10.	1:250	Yes	No	Yes	No	Class I	IgG
12.	1:20	No	Yes	No	Yes	Class II	IgG
13.	1:500	No	No	No	No	Non-HLA	IgM

[a] SOURCE: (5).

TABLE 2. Specificity of cytotoxic and flow cytometric donor-specific antibodies and their immunoglobulin classes in current sera of renal transplanted patients

Pts.	Absorption with HLA class I–coated beads	Absorption with HLA class II–coated beads	Immunoglobulin class	Clinical outcome of graft at 1 yr
1[a]	+	–	IgG	Graft lost within 1 month
2[a]	+	+	IgG	Graft lost after 1 month
3	+	–	IgG + M	Early acute rejection
4[a]	+	+	IgG + M	Graft lost after 3 weeks
5	–	+	IgM	Functioning
6	+	–	IgG	Early acute rejection
7	+	–	IgM	Early acute rejection
8	–	+	IgG	Functioning
9	–	–	–	Graft lost after 1 month
10[a]	+	+	IgG + M	Graft lost after 2 weeks
11	–	–	–	Functioning
12	+	+	IgG	Early acute rejection
13	–	–	–	Graft lost after 3 months
14	–	+	IgM	Functioning
15	+	–	IgG + M	Early acute rejection
16	+	+	IgG	Functioning
17	–	+	IgM	Functioning
18	+	+	IgG + M	Graft lost after 2 weeks
19	+	–	IgM	Early acute rejection
20	–	–	–	Functioning

[a] Patients with weakly positive (15–25%) B cell cytotoxic crossmatches.

mercial availability of HLA antigen-coated beads is sure to find wide application in transplantation immunology laboratories. This method may be further developed to be adapted to a magnetic bead antigen capture enzyme-linked immunosorbent assay (MBAC-ELISA), for use in laboratories with no access to a flow cytometer. We have, in fact, used this approach satisfactorily.

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ANTIBODY SCREENING BY ENZYME-LINKED IMMUNOSORBENT ASSAY USING POOLED SOLUBLE HLA IN RENAL TRANSPLANT CANDIDATES

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The enzyme-linked immunosorbent assay (ELISA) using HLA class I molecules purified from pooled platelets has the potential to detect HLA antibodies with increased efficiency without sacrificing sensitivity or specificity. This test, which was originally developed in our institution, has been independently validated by recent studies and is now commercially available. We now present evidence of its usefulness as a routine HLA antibody screening test for renal transplant patients. A total of 515 patients were tested monthly by ELISA (13.9 tests/patient) and by antiglobulin-enhanced panel reactivity (6.3 tests/patient). In patients found to be unsensitized, the incidence of false-positive results was less for ELISA than for the panel studies. In patients who were highly sensitized, both tests performed equally well, whereas discordant results were registered mainly in cases of mild sensitization. Because 66% of our patients were not sensitized, the ELISA was effective in reducing the number of more involved tests aimed at characterizing the antibodies. These results provide a foundation to use the pooled platelet HLA ELISA on a routine basis for HLA antibody screening.

The presence of anti-HLA class I antibodies (Abs*) specific for transplant donor antigens represents a significant risk for kidney transplant rejection. Knowing beforehand whether the patient has alloantibodies helps to avoid donors bearing the target HLA antigens, to interpret cross-match results at the time of transplantation, and even to select the most appropriate cross-match tests (*1*). The standard technique for Ab screening is the panel reactive antibody (PRA) test, in which a patient's serum is tested with a panel of 30–60 cells by complement-dependent cytotoxicity (CDC). Screening, however, has to be done periodically because either patients may receive blood transfusions or preexisting Abs may decrease over time.

In the last few years, it has become apparent that more efficient Ab screening strategies are needed. On the one hand, health insurance programs and individual laboratories have been burdened with a continuously increasing number of transplant candidates. The number of patients in the renal transplant waiting lists doubled from almost 14,000 in 1988 to about 27,500 in 1994 (*2*). On the other hand, the proportion of patients with preformed Abs has steadily decreased from 40% to 25% in the same period (*2*), probably due to the wider use of erythropoietin and a decrease in blood transfusions. Testing a growing and largely unsensitized patient population with multiple target cells becomes inefficient and wasteful.

A few years ago, an enzyme-linked immunosorbent assay (ELISA) using purified HLA class I molecules from multiple donor platelet pools was developed at our institution (*3*). This technique was sensitive, highly specific for HLA Abs, and correlated well with CDC (*3*). These results were recently independently confirmed (*4*, *5*). The test has subsequently been made commercially available and approved for clinical use (the manufacturer, GTI, Brookfield, WI, has no relationships with our institution or investigators). We have used the ELISA for over 3 years to screen for HLA Abs, along with the standard PRA test. The results reported here represent a large-scale, single-center evaluation of the pooled platelet-soluble HLA ELISA in serial specimens in comparison with findings obtained in panel studies using the anti-human globulin (AHG)-enhanced CDC method.

MATERIALS AND METHODS

Patients. The study included new patients admitted into our renal transplant program and patients already on the cadaveric donor waiting list. Most patients were tested on a monthly basis. Data for this study were collected from July 1993 through November 1995, or earlier if the patients received transplants. In general, sera were tested first by ELISA and then, if positive, by PRA test. However, there were frequent exceptions dictated by individual patient situations in which one or both tests were done in a different sequence. In some cases, testing was also done by flow cytometry. Platelet absorption was performed twice with stored platelets from large donor pools (Scantibodies, Santee, CA). Equal volumes of serum and platelets were incubated for 2 hr, and the serum was recovered by centrifugation.

ELISA. Details of the technique have been published before (*3*). Purified soluble HLA antigens were provided by Dr. K.J. Kao (Department of Pathology, University of Florida, Gainesville, FL). Briefly, HLA antigens were purified by affinity chromatography from platelet pools from over 200 donors. The purified soluble HLA was used to coat the wells of microtiter plates, to which the patient's serum was added. After incubation and washing, IgG Abs attached to the bottom of the well were revealed by appropriate enzyme-labeled second Abs and enzyme substrates. Each serum sample was used at a 1:2 dilution in phosphate-buffered saline and tested in duplicate. Results were expressed as the ratio between the optical density of the patient's serum and that of a normal control serum. Based on the results of tests of multiple normal sera (*3*), a ratio of 1.8 or higher was considered positive. Serum from a broadly sensitized patient at three different dilutions was placed in different parts of the plate and used as a positive control.

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* Abbreviations: Ab, antibody; AHG, anti-human globulin; CDC, complement-dependent cytotoxicity; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; PRA, panel reactive antibody.

PRA test. Panel studies were performed using AHG in a panel of 30 selected cells. The AHG reagent was a goat anti-κ Ab (One Lambda, Canoga Park, CA) used at the optimal dilution to enhance CDC. Some specimens were also tested for IgM in the presence of dithiothreitol or for IgG by flow cytometry. A PRA test was considered positive when it was 10% or higher.

Flow cytometry. Selected specimens were tested by two-color flow cytometry using a panel of 10 random target cells (*6*). Staining and analysis were performed as described elsewhere (*6*, *7*). The test was considered positive if one or more panel cells were positive for IgG T-cell antibodies.

Definition of allosensitization. (a) An isolated, positive ELISA or PRA test, usually weak, with negative results the previous and following month, was regarded as a false-positive result. (b) Patients were considered Ab positive when at least two ELISA or AHG PRA tests were positive. (c) A patient who had Abs detected by both methods was considered allosensitized, as this was strong evidence for the presence of HLA Abs. (d) Patients in whom either the ELISA or PRA test was positive were considered allosensitized if one of the following factors were present: Abs detected by a flow panel, test turned negative after platelet absorption, the patient had unambiguous HLA Abs before the study period, or the patient received blood transfusions proximate to the testing date. (e) Conversely, patients were considered not allosensitized if the flow panel was negative, a positive PRA turned negative in the presence of dithiothreitol, or the patient had no history of blood transfusions, pregnancies, or transplants. (f) Patients negative for both the ELISA and AHG PRA test were considered not allosensitized regardless of previous alloimmunization history.

RESULTS

Incidence of sensitization. We performed 7184 ELISA tests in 515 patients (13.9/patient) and 3232 PRA tests (6.3/patient). Overall, 34% of the patients were sensitized as defined by two or more positive results by one technique or the other (Table 1). As a group, patients who received transplants were less sensitized than those remaining on the waiting list. Table 1 also shows that the proportion of PRA-positive tests was significantly higher than the proportion of ELISA-positive tests. These data indicate that the policy of performing a PRA in samples selected by ELISA is effective in reducing the number of panel studies that are unnecessary because no Abs are present.

Incidence of false-positive results. False-positive results were defined as isolated positive tests in patients considered unsensitized because of multiple negative ELISA and PRA results. The incidence of false-positive results was higher for AHG PRA tests than for ELISAs (Table 2).

Evaluation of Ab-positive patients. Of the 176 patients shown in Table 1 as sensitized by one technique or the other, 154, or 88%, were positive by both ELISA and AHG PRA test. Broadly sensitized patients were always concordant, as results were highly positive by both techniques. Discordant results were seen mostly in patients who developed weakly positive reactions for short periods of time, such as in response to sporadic blood transfusions. In 10 of the 22 discordant cases, Abs were detected by ELISA but not PRA test, and in three cases Abs were detected by PRA test but not ELISA (Table 3). These 13 patients were apparently allosensitized. Nine patients were not considered allosensitized: five of them had a positive PRA/negative ELISA due to the presence of IgM non-HLA Abs, and in four patients, positive ELISA results could not be confirmed due to HLA Abs.

Identifying allosensitized patients. In Table 4, allosensitization was defined not only by ELISA and PRA testing, but also by additional test results and patient history. Because patients were considered Ab positive when the ELISA and/or PRA test was positive, this test combination offers 100% sensitivity. The specificity, however, is 95% because of the nine patients with either IgM non-HLA Abs or no history of allosensitization as described in Table 3. If we require both tests to be positive, the sensitivity decreases to 92% but the specificity increases to 100%. A positive ELISA test, regardless of PRA results, has a sensitivity and specificity of 98%, whereas a positive PRA test using AHG alone has a sensitivity of 94% and a specificity of 97% (Table 4).

Testing by ELISA and PRA on the same sera. When both tests were performed in the same serum sample, concordant results were obtained in 80% of the cases, whereas results were positive by one test but not the other in the remaining 20% (Table 5). Positive results were more frequent by ELISA than by PRA test. Since these patients were sensitized at one time or another, the results suggest that most positive ELISA tests were true positive rather than false positive.

DISCUSSION

The ELISA methodology offers the potential of increased efficiency through reduced hands-on time and automation, increased specificity through the use of purified HLA antigens, and improved reproducibility. At this time, two variants of the ELISA technique are approved for clinical use. One utilizes HLA class I antigens purified from individual cell lines to construct panels of multiple targets, each containing the HLA antigens from a donor rather than whole cells (PRA-STAT; Nextran, Deerfield, IL). This test has good reproducibility (*8*), but the panel approach increases the cost and negates the efficiency advantages of a screening test. Recent reports also question some performance features of this test (*5*, *9*). The other technique utilizes HLA antigens from platelets pooled from a large number of donors (QuikScreen; GTI). This technique was originally developed by K.J. Kao in our institution (*3*) and has been routinely used in our clinical laboratory for several years. The results presented in this article represent the first experience of serial testing with the pooled platelet HLA ELISA and AHG PRA test in individual renal transplant candidates.

About two thirds of the patients we tested were repeatedly

TABLE 1. Incidence of sensitization

Tx	Patients			ELISA			PRA		
	All	Ab+	% Pos	All	Ab+	% Pos	All	Ab+	%Pos
Total	515	176	34	7184	2397	33[a]	3232	2038	63[a]
No	334	132	40	4810	1888	39[a]	2387	1611	67[a]
Yes	181	44	24	2374	509	21[a]	845	427	51[a]

[a] $P<0.000001$, chi-square test.

TABLE 2. False-positive tests in antibody-negative patients

Tx	ELISA			PRA			$P(\chi^2)$
	All	Ab+	%Pos	All	Ab+	%Pos	
Total	4787	183	3.8	1194	112	9.4	0.000001
No	2922	121	4.1	776	85	10.9	0.000001
Yes	1865	62	3.3	418	27	6.5	0.002

TABLE 3. Antibody-positive patients: discordant cases

Test	Circumstances	No. of patients
PRA false negative		10
	Flow panel positive	4
	Transfused proximate to testing	3
	ELISA neg after platelet absorption	2
	History of sensitization	1
ELISA false negative		3
	Flow panel positive	1
	Transfusions proximate to testing	2
PRA false positive		5
	Reduced by dithiothreitol and/or flow neg	5
ELISA false positive		4
	Flow panel negative	3
	No alloimmunization events	1
Total		22

Ab negative, which appears to be representative of nationwide statistics (the United Network for Organ Sharing reported that about 75% of the patients on the 1994 waiting lists had a PRA of <20%) (*2*). Also, of all ELISA tests performed, 67% were negative (Table 1). In contrast, only one third of tests performed using AHG were negative, which is the consequence of performing panel studies preferentially in ELISA-positive specimens.

Is it necessary to do further testing in patients who are repeatedly ELISA negative? Of all patients serially negative for ELISA (n=342 [339 from Table 1 plus 3 from Table 3]), three, or 0.9%, were AHG positive. These three patients appeared to be allosensitized (one patient was multiparous and polytransfused, with a persistent PRA of 20–50%, and the other two patients had received transfusions proximate to testing). In comparison, of all patients serially negative for AHG (n=349 [339 form Table 1 plus 10 from Table 3]), 10, or 2.8%, were ELISA positive and probably allosensitized. These results indicate that the vast majority of ELISA-negative patients are truly unsensitized and do not need further tests. Patients with a history of alloimmunization may be tested the first time using AHG, but, if negative, they can be safely followed with ELISA only. The ELISA also had the advantage of fewer sporadic false-positive results than AHG in unsensitized patients.

In terms of recognizing allosensitized patients by serial testing, the sensitivity and specificity of the ELISA were as good or better than those of the AHG PRA test (Table 4). When individual sera were tested using both methods, the ELISA detected more positive reactions than AHG (Table 5). The ELISA-positive reactions occurred in apparently allosensitized patients and were most likely caused by HLA Abs. Thus, while we cannot conclude that the ELISA is more sensitive than AHG because we selected ELISA-positive sera for AHG testing, these and previous (*3–5*) data indicate that the sensitivity of the ELISA is acceptable and well within the limits of clinical usefulness.

The issue of sensitivity is, moreover, a complex and interesting one. In a previous study, four serum samples with high PRA were tested at several dilutions by ELISA and direct CDC (prolonged incubation). The ELISA detected Abs at 5, 6, 6, and 11 double dilutions higher than CDC (*3*). Although AHG PRA testing was not performed, AHG would have been expected to improve the prolonged incubation sensitivity by only a few dilutions. In contrast, in another study, two monospecific reagent serum samples were positive at higher dilutions by AHG PRA testing than by ELISA (*5*). Thus, it appears that AHG is most sensitive for Abs of narrow specificity, probably because a high proportion of the total Ab content binds to the lymphocytes from a donor bearing the target HLA antigen. When sera have broad specificity, a lower proportion of the total Ab content binds to a given target cell. The ELISA, in contrast, captures most broadly reactive Abs through the multiple specificities present in the antigen preparation. In the present study, most cases of Abs detected by ELISA but not AHG PRA testing were found in

TABLE 4. Antibody-positive patients: allosensitized and not allosensitized[a]

	n	Patients		Test sensitivity (%)	Test specificity (%)
		Allosensitized	Not allosensitized		
ELISA + and/or AHG pos	176	167	9	167/167 (100)	167/176 (95)
ELISA + AHG pos	154	154	0	154/167 (92)	154/154 (100)
ELISA + alone[b]	168	164	4	164/167 (98)	164/168 (98)
AHG + alone[c]	162	157	5	157/167 (94)	157/162 (97)

[a] To define allosensitization, other test results (flow panel, dithiothreitol, platelet absorption) and alloimmunization history were taken into account in addition to ELISA and PRA test results.
[b] AHG positive or negative.
[c] ELISA positive or negative.

TABLE 5. Antibody-positive patients: concordance rate between ELISA and PRA when tested in the same specimen

ELISA	PRA	n	%	Concordance (%)
+	+	937	72	
+	–	106	8	
–				80%
	–	171[a]	13	
+	+	98[a]	7	
–				
Total		1312	100	

[a] $P<0.000001$, chi-square test.

patients after random donor blood transfusions, which are likely to induce Abs of low-level and multiple-epitope specificity. Thus, ELISA or AHG PRA testing may be more sensitive depending on the nature of the Abs present in the test serum.

The results of this study demonstrate that the ELISA test using purified HLA from pooled platelets is a useful tool for HLA Ab screening. Its major advantage is that it identifies the vast majority of unsensitized patients, thus avoiding more expensive screening procedures. It is also very sensitive and specific for detecting HLA Abs, allowing further testing when necessary. Nevertheless, the ELISA does not provide a yes or no answer that can by itself appropriately classify all patients. A correct test interpretation and decision-making process will still require consideration of the patient history of alloimmunizations, the predicted impact that the results obtained may have on a future cross-match, and the potential clinical significance of the Abs detected as it applies to individual patients.

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